The role of reactive oxygen species in the endothelium derived hyperpolarising factor response

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Abstract:
The role of reactive oxygen species in the endothelium derived hyperpolarising factor response

EDHF is the endothelium-dependent but nitric oxide (NO) and prostacyclin (PGI_2) - independent vasodilatation pathway that is the dominant relaxation mechanism in the microcirculation, and has been shown to require IK_{Ca} and SK_{Ca} channel activation and also functional myoendothelial gap junctions. Reactive oxygen species (ROS) have been implicated in the response, but their precise role remains uncertain. To investigate this, the cremaster muscle circulation of freshly killed rats was perfused with a Krebs buffer solution containing albumin (1 $g.l^{-1}$) and either FITC-albumin (2 $g.l^{-1}$) to measure changes in diameter, or Fura-PE3AM (10 μM) for 60 minutes to selectively load the endothelium, allowing endothelial $[Ca^{2+}]_i$ to be recorded. The preparation was placed on the stage of an intravital microscope to measure vessel diameter, and endothelial $[Ca^{2+}]_i$ was estimated from the 360/380 nm excitation ratio, emission at > 510 nm. The preparation was superfused with phenylephrine (30 μM), L-NAME (300 μM) and indomethacin (3 μM) to evoke arteriolar constriction while preventing the synthesis of NO and PGI_2. Carbachol (10 μM) caused a 74.5 ± 2.3% (n = 65) relaxation and the 360/380 nm ratio increased by 25.6 ± 1.6% (n = 69). Both responses were substantially reduced by a ROS scavenging combination of superoxide dismutase and catalase (100 $U.ml^{-1}$ each). The possibility that ROS are important for Ca^{2+} release from stores was examined by applying carbachol in Ca^{2+}-free solution containing EGTA. Carbachol application then resulted in a transient $[Ca^{2+}]_i$ increase that was reduced by SOD and catalase. The CYP 2C9 inhibitor sulfaphenazole (10 μM) reduced relaxation and the endothelial $[Ca^{2+}]_i$ increase, as did the phospholipase C blocker U73122. These and other data to be presented suggest that ROS produced by arachidonic acid metabolism via CYP 2C9 promote EDHF

mediated relaxation mainly by enhancing the inositol trisphosphate-mediated release of Ca^{2+} from endothelial stores.

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Most of all I would like to thank my family for their unwavering love and support.

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Declaration

All the work presented in the following thesis has been performed by James Chidgey other than the electrophysiology work which was carried out with Dr Kim Dora and Dr Timea Beleznai at the University of Oxford, UK.

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List of abbreviations

18α-GA	18 alpha-glycyrrhetinic acid
ATP	Adenosine triphosphate
BH_4	Tetrahydrobiopterin
BK_{Ca}	Large conductance Ca^{2+} activated K^+ channels
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CNP	C-natriuretic pepetide
**COX ` **	Cyclooxygenase
CPA	Cyclopiazonic acid
CYP450	Cytochrome P450
CRAC	Ca^{2+} release activated Ca^{2+}
DFO	Desferrioxamine
EGTA	Ethylene glycol tetraacetic acid
DAG	Diacylglycerol
DCF	Dichlorofluorescein
DHE	Dihydroethidium
DMSO	Dimethylsulphoxide
EC	Endothelial cell
EDH	Endothelium dependent hyperpolarisation
EDHF	Endothelium derived hyperpolarising factor
EDRF	Endothelium derived relaxing factor
EETs	Epoxyeicosatrienoic acids
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
IK_{Ca}	Intermediate conductance Ca^{2+} activated K^+ channels
IL-1β	Interleukin-1beta
IP_3	Inositol trisphosphate
IP_3R	Inositol trisphosphate receptor

K_{ATP}	ATP sensitive K^+ channels
K_{Ca}	Ca^{2+} activated K^+ channels
K_{IR}	Inwardly rectifying K^+ channels
KO	Knock out
K_V	Voltage-dependent K^+ channels
L-NAME	L-N^G-Nitroarginine methyl ester
L-NNA	Nω- Nitro-L-arginine
LOX	Lipoxygenase
MEGJ	Myoendothelial gap junction
MLC	Myosin light chain
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	NADPH oxidase
NPR	Natriuretic peptide receptor
O_2^-	Superoxide
$^{\cdot}OH$	Hydroxyl radical
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PKA	Protein kinase A
PKG	Protein kinase G
PSS	Physiological saline solution
ROS	Reactive oxygen species
sEH	Soluble epoxide hydrolase
SK_{Ca}	Small conductance Ca^{2+} activated K^+ channels
SMC	Smooth muscle cells
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
TEA	Tetraethylammonium
TNFα	Tumour necrosis factor alpha
TPR	Total peripheral resistance

TRP	Transient receptor potential
VDCC	Voltage dependent Ca^{2+} channels
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cell
X/XO	Xanthine/Xanthine oxidase

Chapter 1: General introduction

1.1 Vascular Physiology

1.1.1 Vascular tone

Blood vessels exhibit vascular tone, a state of partial constriction from which they can either dilate or further constrict. The intravascular pressure which drives the flow of blood is highly dependent on vascular diameter (pressure is inversely proportional to the 4^{th} power of the diameter). Therefore, local changes in vascular tone exert a profound effect on blood flow within tissues, and at the level of the entire system the vascular tone of the arteries and arterioles determines the total peripheral resistance and the arterial blood pressure (arterial blood pressure = cardiac output x total peripheral resistance) (Levick, 2010).

The regulation of vascular tone in arteries (hereafter the term arteries will be used to encompass both arteries and arterioles except where noted) is influenced by the architecture of the individual vessels. Arteries are composed of three main layers, the intima, media and adventitia (Tharp & Bowles, 2009). The intima is the inner layer that surrounds the lumen through which the blood flows. The lumen of the entire cardiovascular system is lined by a monolayer of endothelial cells. The endothelium not only acts as a semi-permeable barrier between the blood and underlying tissue and governs tissue exchange, but is also vital in the regulation of the cardiovascular system through releasing factors that can act locally and systemically (Sanders et al., 2000). The endothelium can sense changes in shear stress from blood flow and has receptors for agonists that can influence vascular tone by causing the release of vasoactive substances. The endothelium can secrete vasodilators such as nitric oxide (NO) and prostacyclin (PGI_2) and vasoconstrictors like endothelin which act on the

underlying smooth muscle. Overall, however, the vasodilating effect of the endothelium predominates over its constricting effect (Carvajal et al., 2000).

The internal elastic lamina separates the intima from the media which is composed of smooth muscle cells and matrix proteins such as collagen and elastin. In larger conduit arteries this smooth muscle layer can be many layers thick whereas in smaller arterioles there may only be a single layer of smooth muscle cells (Levick, 2010).

Finally there is the adventitia, which is separated from the media by the external elastic lamina. The adventitia is composed of fibroblasts and loosely arranged collagen, and contains sympathetic nerves fibres which release noradrenaline, the most important physiological vasoconstrictor.

My project examined the regulation of vascular tone in smaller resistance vessels of the rat cremaster circulation. The resistance vessels include the smaller arteries and arterioles that are the major determinants of total peripheral resistance. Arterioles give rise to capillaries where oxygen and nutrient exchange takes place in tissues and are part of the microcirculation. Arterioles have diameters of 10-100μm and dominate the resistance to flow (Levick, 2010). While constriction of a large proportion of arterioles would increase TPR, local contraction can regulate blood flow to tissue and organs by directing blood flow away from areas of greater resistance (Aaronson & Ward, 2007). Regulation of vascular tone in resistance vessels, primarily by the endothelium acting on smooth muscle cells, is therefore important for local blood flow as well as overall blood pressure.

1.1.2 Ca^{2+} regulation

Vascular tone is determined by the degree to which the smooth muscle cells (SMCs) in the media are contracted. These elongated spindle-shaped cells wrap around blood vessels in a helical manner, so as they shorten and thicken the vascular diameter is reduced. The major determinants of contraction in SMC are the cytoplasmic Ca^{2+} concentration, and Ca^{2+} sensitisation (Ignarro, 2002).

1.1.3 Regulation of $[Ca^{2+}]_i$ in VSMC

Ca^{2+} is an important second messenger in many types of cells, and provides one of the main pathways by which extracellular signals can be transduced to intracellular sites. The location, concentration and duration of the Ca^{2+} rise defines the signal that is transferred. In the vasculature, many endothelial and smooth muscle cell responses are triggered by changes in cytosolic Ca^{2+} concentration, including the release of vasoactive substances and changes in membrane permeability (Levy, 2005).

The intracellular basal cytosolic Ca^{2+} concentration is approximately 0.1µM while the extracellular concentration is about 1.5 mM. This leads to a large electrochemical Ca^{2+} gradient across the plasma membrane (Levick, 2010). Cytosolic $[Ca^{2+}]$ can increase following the influx of extracellular Ca^{2+} through ion channels in the membrane, or by release of Ca^{2+} from intracellular stores. There are a range of Ca^{2+} channels whose expression in different cell types varies, and there are different pathways that regulate their activity. Intracellular $[Ca^{2+}]$ normally increases following an extracellular signal such as the binding of an agonist to a receptor on the plasma

membrane. This can then cause activation of ion channels on the plasma membrane and release of calcium from intracellular stores (Ohana et al., 2009).

1.2 Ca^{2+} Channels

1.2.1 Plasma membrane Ca^{2+} channels

The influx of extracellular Ca^{2+} can occur through a number of cation channels that are activated by different signals such as membrane depolarisation, shear stress or agonist binding to receptors, or from intracellular signalling following the depletion of the sarcoplasmic reticulum, an intracellular Ca^{2+} store (Webb, 2003).

1.2.2 Voltage dependent Ca^{2+} channels (VDCC).

Voltage-dependent or -gated Ca^{2+} channels are found in a range of excitable cells including vascular SMCs, where they are important in Ca^{2+} regulation (Marrelli, 2000). There is little evidence of their expression in endothelial cells. Their opening is voltage dependent, and at resting membrane potentials the channels are mainly closed. They open as a result of membrane depolarisation, causing calcium influx down the electrochemical gradient. This increase in intracellular calcium can then cause the activation of different calcium dependent pathways including release of regulatory factors, up-regulation of gene expression, and muscle contraction. They also allow entry of sodium ions but they have 1000 fold greater selectivity for Ca^{2+} (Levick, 2010). There are different families of voltage dependent Ca^{2+} channels and the L-type

calcium channel is the form mainly found in vascular smooth muscle cells (Aaronson & Ward, 2007).

1.2.3 VSMC membrane potential and its control of VDCC

The open probability of L-type VDCC is determined by the membrane potential. Under resting conditions, most but not all of these channels are closed, so they mediate a small tonic Ca^{2+} influx (Bychkov et al., 2000).

The resting membrane potential in vascular smooth muscle is -60 to -75 mV and -40 to -55 mV when measured *in vitro* and *in vivo*, respectively (Nelson et al., 1993). This negative potential is thought to be partly due to large negatively charged molecules, mainly proteins, present in the cytosol. The membrane is somewhat permeable to K^+, which is drawn into cells by this negative charge. This would cause its concentration within cells to become higher than it is outside, until it reached a concentration at which its electrochemical gradient would be in balance (i.e. its tendency to move out of cells due to this high concentration would be exactly balanced by its tendency by its tendency to enter cells due to the negative intracellular charge). K^+ is also actively accumulated by the Na^+/K^+ ATPase, which pumps 2 K^+ ions into the cell while extruding 3 Na^+ ions. These factors mean that the K^+ concentration in cells is much higher (~130 mM) than outside (~4 mM) (Aaronson & Ward, 2007). As a result, the opening of K^+ channels in the cell membrane leads to an efflux of K^+ ions, and this movement of positive charge out of cells drives the membrane towards the K^+ equilibrium potential, which is close to -90 mV. However, the membrane potential is

less negative than this because the plasmalemma is also permeable to Na^+ and Cl^- which have more positive reversal potentials.

The result of this arrangement is that the opening of K^+ channels, and also stimulation of the Na^+/K^+ ATPase, causes the membrane to hyperpolarise towards -90 mV. This diminishes the open probability of L-type channels, leading to a fall in Ca^{2+} influx and vasorelaxation (Bellien et al., 2008). This regulation of membrane potential and therefore Ca^{2+} influx by K^+ channels and the Na^+/K^+ ATPase is important in the control of VSMC by EDHF (see section 1.7.4).

1.2.4 Receptor operated calcium channels.

Receptor operated calcium channels are activated by the binding of a specific molecule to a receptor, for example G protein coupled receptors such as the α_1 adrenergic receptor in vascular SMC. They vary in their relative selectivities to Ca^{2+} ions and Na^+ ions, and in smooth muscle are thought to mainly mediate Na^+ influx, leading to cell depolarisation and the opening of voltage-gated Ca^{2+} channels (de Wit, 2010). The molecular identities have not been clearly defined and different candidates have been put forward as being part of this group, for example transient receptor potential (TRP) channels such as TRPC1, C3, C6 and C7 have now all been shown to be activated by DAG following ligand binding to G protein coupled receptors (Inoue et al., 2001)

1.2.5 Store operated calcium channels.

When the endoplasmic reticulum in endothelial cells, or the sarcoplasmic reticulum in smooth muscle cells, is depleted of Ca^{2+}, store operated channels in the plasma membrane open. This influx of calcium is called store operated or capacitative Ca^{2+} entry and helps to replenish the stores and may also be involved directly with Ca^{2+} signalling. The plasma membrane channel involved in some types of cells (e.g. lymphocytes) is referred to as the Ca^{2+} release activated Ca^{2+} (CRAC) channel, and it is the decrease in calcium concentration in the lumen of the organelles that leads to its activation. This is a major calcium influx pathway in some cells (for example endothelial cells) so is important in signal transduction (Alaiwi et al., 2009). 2 main protein families are recognised as being important in the function of store operated Ca^{2+} entry, STIM and Orai. The stromal interaction molecule (STIM) is a single spanning transmembrane protein. STIM functions as a Ca^{2+} sensor on the endoplasmic reticulum and following Ca^{2+} depletion rearranges to a site near the plasma membrane where it interacts with the CRAC channel. STIM1 and 2 expression have been demonstrated in endothelial and vascular smooth muscle cells and they have differing affinities for Ca^{2+} but STIM1 is thought to mainly control the CRAC current (Smyth et al., 2010). The Orai channel family form the pore of the CRAC channel. Orai 1-3 are 4 transmembrane spanning proteins which form a highly Ca^{2+} selective channel. Following Ca^{2+} store depletion the Orai channel subunit is activated by STIM1 leading to Ca^{2+} entry (Putney, 2011). The TRP channel subfamily TRPC, and in particular TRPC1 have been suggested to be involved in CRAC current, forming a separate channel to the Orai family. It has been suggested that Orai

channels may be involved with the initial phase of Ca^{2+} entry while the TRPC1 channel may allow sustained entry (Smyth et al., 2010).

1.2.6 Transient receptor potential (TRP) channels

TRP channels are a group of non-selective cation channels and they can function as sensors for internal and external stimuli (Kwan et al., 2007). There are around 28 TRP channels which have so far been identified, including the TRPC, TRPV and TRPM channel families. They have been suggested to be involved in receptor and store operated calcium entry (Beech, 2005). There are a number of TRP channels that are mainly located on the plasma membrane of nearly all cell types including endothelial cells and SMC. The cation channels have a varying selectivity for calcium vs other cations such as Na^{+} (Venkatachalam & Montell, 2007). The TRP channels share similar structural features such as 6 transmembrane spanning domains with intracellular N and C terminals. They are activated by range of stimuli including shear stress, temperature changes and agonists (Mendoza et al., 2009). They appear to be important ion channels for acting as sensors for changes in the environment such as pressure and flow. The 6 subfamilies are TRPC, TRPV, TRPM, TRPA, TRPP and TRPML. The channels are made up of 4 subunits which can be formed from the same type, homomeric, or from different subunits and be heteromultimeric which alters its function.

The TRPC (canonical) subfamily has 7 members (C1-C7) but TRPC2 is not functional in humans. They are Ca^{2+} permeable but not Ca^{2+} selective and are not voltage gated. TRPC1 has been suggested to be involved in store operated Ca^{2+} entry,

either on its own or by forming a complex with Orai channels (Song et al., 2011). TRPC1, C3, C6 and C7 also act as receptor operated Ca^{2+} channels through direct activation by DAG following binding of a ligand to a G protein coupled receptor. For example Ca^{2+} entry through TRPC1 on vascular smooth muscle cells can cause constriction following binding of endothelin to a GPCR (Earley & Brayden, 2010).

The TRPV (vanilloid) subfamily has 6 members with TRPV1, V3, V4 and possibly V2 present in endothelial cells and TRPV2 and TRPV4 found to be functional in vascular smooth muscle cells. TRPV1 is activated by capsaicin, the active ingredient in chilli peppers, while V2 is involved in the sensation of thermal pain (Earley & Brayden, 2010). While TRPV5 and V6 are constitutively active and Ca^{2+} selective, TRPV4 on vascular smooth muscle can be activated by EETs, leading to Ca^{2+} entry and activation of the BK_{Ca} channels causing hyperpolarisation and relaxation (Di & Malik, 2010).

The TRPM (melastatin) subfamily has 8 members with varying selectivities. While TRPM1 and M2 are non-selective cation channels, M6 and M7 are selective for Mg^{2+} and are important in its homeostasis. All the TRPM channels other than M5 have been shown to be expressed in endothelial cells but only TRPM2 has been shown to be functionally active (Earley & Brayden, 2010). All of the members are expressed in vascular smooth muscle cells with TRPM4 thought to be an important mechanosensor contributing to myogenic tone by causing vasoconstriction in response to pressure (Vennekens, 2011).

The TRPA (ankyrin) subfamily only has one member, TRPA1. This has been shown to be present in cerebral arteries and is activated by electrophilic compounds, including those in mustard oil and garlic and it may mediate the endothelium dependent relaxation to these compounds through the activation of the IK_{Ca} and SK_{Ca} channels (Earley & Brayden, 2010).

The TRPP (polycystic) and TRPML (mucoliptin) subfamilies each have 3 members and have not been shown to be active in the cardiovascular system. TRPP1 mutations cause polycystic kidney disease while TRPML channels are important in lysosome function (Earley & Brayden, 2010).

1.2.7 Endoplasmic /sarcoplasmic reticulum Ca^{2+} channels.

A major pathway in causing an increase in intracellular [Ca^{2+}] during stimulation is its release from intracellular stores (the endoplasmic or sarcoplasmic reticulum) following agonist binding to Gq protein coupled receptors which are linked to membrane bound phospholipase C. Examples are the muscarinic M_3 receptor in the endothelial cells and the α_1 adrenergic receptor in vascular SMCs. Following binding by an agonist such as acetylcholine, the G protein acts to cause the release of second messengers. The M_3 receptor is coupled to Gq which activates phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2), a phospholipid in the cell membrane, into the second messengers IP_3 and DAG (Levick, 2010). DAG stays within the membrane (where it can, for example, activate TRPC channels (Venkatachalam et al., 2007) whereas IP_3 is soluble and can diffuse to the ER/SR and bind to its receptor. The IP_3 receptor is a membrane glycoprotein complex

that acts as a Ca^{2+} channel following its activation and causes Ca^{2+} release from the stores. This causes an increase in cytoplasmic Ca^{2+} concentration (Takahashi et al., 2008). As described above, this store depletion can lead to the activation of store operated Ca^{2+} channels on the surface membrane and therefore allow influx of extracellular Na^{+} and Ca^{2+}.

1.2.8 Ryanodine receptor

The ryanodine receptor is another Ca^{2+} channel present on intracellular membranes including the sarcoplasmic reticulum in SMCs and other excitable cells such cardiac myocytes and neurons. The receptors are similar to the IP_3 receptor and activated by increases in cytoplasmic [Ca^{2+}] from IP_3 mediated release or influx of extracellular Ca^{2+}. This process is known as Ca^{2+}-induced Ca^{2+} release and acts as a positive feedback mechanism causing a further Ca^{2+} release into the cytosol (Carvajal et al., 2000). In skeletal muscle the activation may occur via direct coupling with a receptor rather than calcium-induced calcium release. The ryanodine receptor is particularly important in the Ca^{2+} signalling that leads to the contraction of skeletal and cardiac muscle.

1.3 How Ca^{2+} causes contraction

The intracellular Ca^{2+} concentration controls vascular smooth muscle contraction and relaxation. An increase in intracellular Ca^{2+} leads to Ca^{2+} binding to the messenger protein calmodulin forming a Ca^{2+}-calmodulin complex. This complex causes the activation of myosin light chain (MLC) kinase which phosphorylates MLC. The

phosphorylated MLC then becomes active and it can form crossbridges with actin which causes the contraction (Carvajal et al., 2000). MLC phosphatase dephosphorylates the myosin filaments which stop the crossbridging between the myofilaments. When the Ca^{2+} concentration is lowered, MLC kinase activity is reduced and the MLC phosphatase activity dominates leading to relaxation (Sandow & Hill, 2000).

1.3.1 Ca^{2+} sensitisation

The contractile state of vascular smooth muscle is also regulated by Ca^{2+} sensitisation as well as the change in intracellular Ca^{2+} concentration (Somlyo & Somlyo, 1994). The monomeric G protein rhoA is activated when agonists bind to G protein coupled receptors, and in turn stimulates its substrate Rho kinase. This can directly phosphorylate MLC allowing crossbridging with actin filaments leading to contractions, but its predominant mode of action is to phosphorylate MLC phosphatase, thus inhibiting its activity. This increases the sensitivity of the contractile apparatus to Ca^{2+} as there is enhanced MLC phosphorylation with the MLC phosphatase inactivated (Gupte et al., 2008). The net effect is to enable contraction with smaller increases in $[Ca^{2+}]_i$.

1.4 Mechanisms of vascular SMC relaxation

Relaxation of the vascular smooth muscle cell occurs when there is a decrease in intracellular $[Ca^{2+}]$, as this leads to a reduction in activation of the MLC kinase by the Ca^{2+}-calmodulin complex and the dominance of the MLC phosphatase. This causes

dephosphorylation of the MLC and prevents crossbridge cycling with actin (Caravajal et al., 2000). This reduction in Ca^{2+} can be from the active efflux of Ca^{2+} out of the cell, its pumping into internal stores and the closure of Ca^{2+} channels. Reduced Ca^{2+} sensitisation also causes relaxation. Different stimuli such as acetylcholine or shear stress lead to relaxation following the activation of receptors which produce a signalling cascade. This produces second messengers or acts through a pathway which ultimately decreases VSM [Ca^{2+}] leading to relaxation (Jackson, 2000).

1.4.1 Ca^{2+} removal from the cytoplasm

The decrease in [Ca^{2+}] in the VSM cell during vasorelaxation is often partly due to the closure of voltage-gated Ca^{2+} channels which inhibits Ca^{2+} influx. This happens following hyperpolarisation of the smooth muscle cell. Ca^{2+} is also actively removed from the cytoplasm by a number of different mechanisms (Somlyo & Somlyo, 1994). There is active Ca^{2+} extrusion via pumps on the cell membrane, as well as sequestration into the intracellular Ca^{2+} stores. Ca^{2+} ATPase ion pumps actively remove Ca^{2+} from the cytoplasm and are present on the plasma membrane and the sarcoplasmic reticulum (Ishida & Paul, 2005).

1.4.2 Plasma membrane Ca^{2+} ATPase

The plasma membrane Ca^{2+} ATPase is an ion pump which actively removes Ca^{2+} into the extracellular space. It is widely expressed in tissue including the VSM cells. Ca^{2+} is being moved against its electrochemical gradient so the pump requires energy from ATP hydrolysis. This allows cell signalling to be regulated as the intracellular Ca^{2+}

concentration is restored towards its basal level, thus permitting it to be increased again (Somlyo & Somlyo, 1994).

1.4.3 Sarcoplasmic reticulum membrane Ca^{2+} ATPase

The sarcoplasmic reticulum membrane Ca^{2+} ATPase (SERCA) is an ion pump on the internal Ca^{2+} store with smooth muscle cells. Following contraction, the SERCA pump actively transports Ca^{2+} against its concentration gradient from the cytoplasm into the sarcoplasmic reticulum. This lowers in the intracellular Ca^{2+} concentration leading to muscle relaxation (Levick, 2010; Ishida & Paul, 2005).

1.4.4 Na^{+}/Ca^{2+} exchanger

The Na^{+}/Ca^{2+} exchanger is an antiporter protein in the plasma membrane. It reduces the intracellular Ca^{2+} concentration by removing Ca^{2+} from the cells. It uses the electrochemical gradient of Na^{+} which is at a higher concentration outside the cell to power the exchange. The energy lost by Na^{+} moving into the cell is used to provide energy for Ca^{2+} extrusion, with one Ca^{2+} ion removed for three Na^{2+} ions entering (Somlyo & Somlyo, 1994). There are 3 main isoforms of the exchanger, NCX with varying expression in different tissues. NCX1 family are the most important in the cardiovascular system with NCX1.1 primarily in cardiac cells, and NCX1.3 in vascular smooth muscle. Along with the Ca^{2+} ATPase pumps this mechanism is responsible for maintaining and restoring the low resting levels of intracellular [Ca^{2+}] in smooth muscle cells (Ishida & Paul, 2005).

1.4.5 VSMC relaxation induced by cyclic nucleotides

Following stimulation by agonists, the pathway that leads to relaxation often involves cyclic nucleotides such as cGMP or cAMP. cGMP is generated by soluble guanylate cyclase which converts GTP to cGMP, and cAMP is synthesised from ATP by adenylate cyclase. The cyclic nucleotides are broken down by phosphodiesterases into inactive products. cGMP and cAMP activate specific protein kinases, PKG and PKA respectively (Somlyo & Somlyo, 1994). These kinases phosphorylate target proteins which leads to a reduction in intracellular Ca^{2+} and relaxation. For example, PKG and PKA can directly open K^+ channels on the smooth muscle leading to hyperpolarisation and closure of voltage-gated Ca^{2+} channels (Jackson, 2005; Aaronson & Ward, 2007). The plasma membrane Ca^{2+} ATPase and SERCA can also be activated directly to increase Ca^{2+} efflux and sequestration into the sarcoplasmic reticulum. These pumps are regulated by phospholamban which is phosphorylated by the kinases and increases activity. IP_3 is a second messenger that is produced by PLC activity and causes release of Ca^{2+} from the sarcoplasmic reticulum. PKG phosphorylates the IP_3 receptor which reduces the Ca^{2+} channel's activity. It also reduces IP_3 production by directly inhibiting PLC and through phosphorylation of the GPCR that stimulates PLC (Caravajal et al., 2000). During contraction there is Ca^{2+} sensitisation which, as described above, increases the contractile state due to MLC phosphatase inhibition. However, PKG stimulates the MLC phosphatase leading to dephosphorylation of MLC and therefore Ca^{2+} desensitisation (Khatri et al., 2001). The cyclic nucleotides therefore cause relaxation by directly lowering intracellular Ca^{2+}, and also by inhibition of the contractile mechanism.

1.5 Hyperpolarisation and K^+ channels

The membrane potential of the cell is important in regulation of vascular tone, as this affects the open probability of the voltage-gated Ca^{2+} channels. A number of types of K^+ channels on the endothelial cells and smooth muscle cells are important in the regulation of the membrane potential (Nelson et al., 2005; Jackson, 2005). The opening of the K^+ channels leads to efflux of the cation down its electrochemical gradient and hyperpolarisation of the cell (Jackson, 2000).

1.5.1 Ca^{2+} activated K^+ channels

The Ca^{2+} activated K^+ channels (K_{Ca}) are ion channels which are stimulated by an increase in intracellular Ca^{2+}. They are categorised by their conductance into BK_{Ca}, IK_{Ca} and SK_{Ca} types (for big, intermediate and small conductance). The BK_{Ca} channels have been found to be widely expressed while IK_{Ca} and SK_{Ca} are predominately found on endothelial cells (Jackson, 2000, 2005). The discovery or development of specific inhibitors for each of the different subtypes has allowed for investigation of their different roles in regulating vascular tone.

1.5.2 SK_{Ca} channels

The SK_{Ca} channel has a conductance of 5-18pS and is opened by 200-500nM $[Ca^{2+}]_i$ (de-Allie et al., 1996). The channel is voltage independent and has been found to be blocked by the peptide apamin from bee venom (Tharp & Bowles, 2009). The channel's Ca^{2+} sensitivity is due to Ca^{2+} binding to calmodulin which is associated with the C terminal; this increases its open probability. It is a tetramer of 4 α subunits each consisting of 6 transmembrane spanning domains that form the pore. There are 3

types of subunits, K_{Ca} 2.1, 2.2 and 2.3 but in endothelial cells K_{Ca} 2.3 is the only one found to be expressed (Edwards et al., 2010).

1.5.3 IK_{Ca} channels

The IK_{Ca} channel has a conductance of 20-80pS and is activated by 100-300nM $[Ca^{2+}]_i$ (Tharp & Bowles, 2009). The IK_{Ca} channel also has a C terminal with calmodulin acting as the Ca^{2+} sensing domain. The channel is inhibited by the scorpion venom charybdotoxin, which also inhibits BK_{Ca} channels, and is specifically blocked by TRAM 34. Like the SK_{Ca} channel it is formed of 4 α subunits which are constitutively associated with calmodulin. It is a homotetramer formed of the K_{Ca} 3.1 subunit (Jackson, 2005).

1.5.4 BK_{Ca} channels

The BK_{Ca} channel has a conductance of 100-200pS and is regulated by membrane potential voltage as well as by $[Ca^{2+}]_i$ (Tharp & Bowles, 2009). The BK_{Ca} channels have a voltage sensor as well as a Ca^{2+} sensing domain at the C terminal. In the vascular system, the BK_{Ca} channels are located primarily on the VSM cells, and are inhibited by iberiotoxin (Gutterman et al., 2005). The BK_{Ca} channels are made of 4 α subunits, from the KCNMA1 gene, that form the pore, and 4 β subunits, from the KCNMB1-4 genes, that modulate the Ca^{2+} sensitivity so calmodulin is not required. Under normal conditions it does not contribute to the resting membrane potential and on vascular smooth muscle cells is primarily activated by Ca^{2+} rather than membrane depolarisation. These channels are activated by the cAMP/PKA signal transduction pathway, for example following stimulation of vascular smooth muscle cells by β-adrenergic receptor agonists, prostacyclin, and adenosine (Jackson, 2005).

1.5.5 Inward rectifying K^+ channels (K_{IR})

K_{IR} channels are K^+ selective ion channels which are so named because they pass an inward K^+ current more easily than an outward current, although at the normal resting potential they, like other K^+ channels, mediate Ca^{2+} efflux (Petersson et al., 1997). They have the unusual property of being stimulated by small increases in extracellular [K^+], which therefore can cause cell hyperpolarisation if these channels are present. They have been found in many cell types including vascular smooth muscle cells and endothelial cells, where they may be involved in the response to shear stress. The $K_{IR}2.1$ is the main isoform in these cells, and like other K^+ channels is formed from 4 α subunits that make a pore. K_{IR} channel expression is greater in smaller vessels which could lead to differing responses between vessels (Jackson, 2000, 2005).

1.5.6 ATP sensitive K^+ channel (K_{ATP})

K_{ATP} channels are inhibited by increases in cellular [ATP] and are present in different membranes of the cell including the mitochondria and plasma membrane (Jackson, 2000). These channels are particularly important in pancreatic β cells, where their inhibition due to a rise in cellular [glucose] leads to depolarisation, a rise in Ca^{2+} influx, and insulin release, but they are also found on other cell types including vascular smooth muscle cells (Gutterman et al., 2005). They are formed from a tetramer of KIR6.1 α subunits that make the pore, and 4 regulatory sulfonylurea receptor (SUR2B) subunits (Jackson, 2005)

1.5.7 Voltage-gated K^+ channels (K_V)

K_V channels are sensitive to changes in the membrane potential, being opened by depolarisation. Like BK_{Ca} channels they contain a voltage sensor and they are important in returning cells to a resting state following depolarisation, and contribute to the resting membrane potential (Tharp & Bowles, 2009). These channels can be directly activated by cAMP leading to hyperpolarisation and relaxation in smooth muscle cells (Gutterman et al., 2005). They are expressed in endothelial cells but their function is not clear. They are formed from a tetramer of KVα subunits which make the ion conducting pore, along with 4 modulatory KVβ subunits. The tetramer is normally homotetrameric and over 40 KVα subunits have been described. In vascular smooth muscle cells, KV1.5 and KV1.6 from the delayed rectifier group appear to be the most widely expressed and in hypertension models there is functional downregulation of these channels (Edwards et al., 2010; Jackson, 2005).

1.6 Endothelium-dependent relaxation

The endothelium can release a number of identified factors which can activate signalling pathways in VSMC leading to vasodilatation. These include nitric oxide (NO), which activates the cGMP/PKG pathway and prostacyclin/PGI_2, which activates the cAMP/PKA pathway. The endothelium also releases the vasoconstricting peptide endothelin, which is not directly relevant to this thesis so will not be considered further.

1.6.1 NO

Endothelium derived NO is recognised as playing a significant role in the control of vascular tone to stimuli such as histamine which increase NO synthesis (Feletou & Vanhoutte 1988; Groschner et al., 1994). Following activation of the endothelial cell and a rise in intracellular [Ca^{2+}], NO is released and diffuses to the vascular smooth muscle cells, where it binds to soluble guanylate cyclase cauings the production of cGMP. This then acts through PKG to cause Ca^{2+} desensitisation, a reduction in the intracellular Ca^{2+} concentration in the smooth muscle (Carvajal et al., 2000; Cary et al., 2006), and vasodilatation. NO and nitric oxide synthase (NOS), in particular endothelial NOS (eNOS) which generates NO in the endothelium, are important in physiological regulation of the vasculature (Vallance et al., 1989). The NO induced relaxation is often found to be inhibited in pathological situations (Sanders et al., 2000).

1.6.2 PGI_2

PGI_2 is another important factor released by the endothelium. It is produced from the breakdown of arachidonic acid by cyclooxygenase (COX), and causes vasodilatation by binding to the G protein coupled prostanoid IP receptor. This stimulates adenylate cyclase, causing production of cAMP (Fetalvero et al., 2007) and the activation of PKA which can activate channels including the K_{ATP} channel to cause hyperpolarisation, thereby causing relaxation of the smooth muscle (Parkington et al., 2004).

1.7 EDHF

Following activation of the endothelial cell by an agonist such as acetylcholine or bradykinin, or by the physiologically more important stimulus of shear stress, there is a rise in intracellular Ca^{2+} levels. This leads to the hyperpolarisation of the endothelial cell and then the subsequent hyperpolarisation of the vascular smooth muscle cell and relaxation (Edwards et al., 2010). It has been shown that the rise in endothelial intracellular calcium leads to the activation of SK_{Ca} and IK_{Ca} channels (Andersson et al., 2000), and it is the efflux of K^+ through these channels that leads to the hyperpolarisation of the endothelial cell. The response is termed endothelial-dependent hyperpolarisation (EDH) and is independent of the NO and PGI_2 pathways that have been described above. The involvement of the two endothelial K_{Ca} channels is a defining characteristic of the EDHF process (Edwards et al., 2010). Inhibitors of the SK_{Ca} and IK_{Ca} channels, apamin and charybdotoxin (or TRAM-34) respectively, are used to selectively inhibit the EDHF response (Doughty et al., 1999).

The exact nature of EDH is not yet known despite extensive research. Originally it was believed that EDH was caused by a substance that was released from endothelial cells and diffused to the vascular smooth muscle to directly cause hyperpolarisation. This substance was christened EDHF (Busse et al., 2002). Research now suggests that EDHF is not due only to a diffusible factor, but is rather a process that leads to hyperpolarisation of the endothelial cell and then the vascular smooth muscle cell, with this latter step probably requiring a diffusible factor and also electrical coupling between the endothelium and VSM, with the balance between these mechanisms dependent on the particular vascular bed and the level of stimulus (Mather et al.,

2005; Parkington et al., 2008). However, for convenience I will hereafter refer to this process as the EDHF response.

1.7.1 The discovery of the EDHF response

The EDHF response was first described more than 20 years ago. It was found that in rat aorta and pulmonary arteries, acetylcholine caused a relaxation that was endothelium dependent, but was independent of COX and NOS (Chen et al., 1988). These enzymes produce PGI_2 and NO respectively, mediators of the main relaxation pathways, so this suggested there was a third endothelium dependent mechanism. In canine coronary artery, this mechanism was found to involve hyperpolarisation which leads to relaxation of the smooth muscle cell (see Fig. 1.1, taken from Feletou & Vanhoutte, (1988).

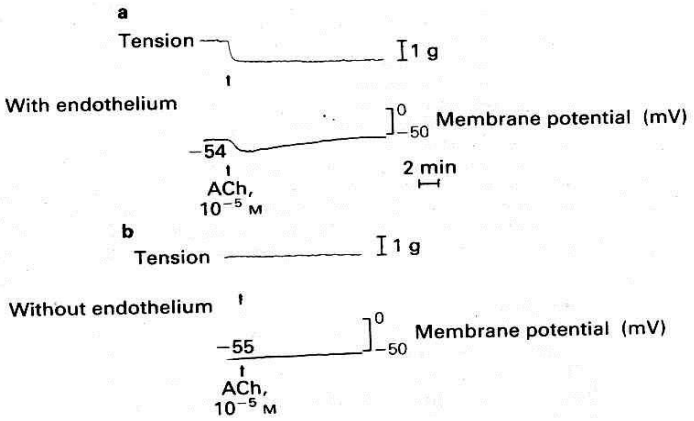

Figure 1.1. Endothelium dependent relaxation and hyperpolarisation to acetylcholine. (Feletou & Vanhoutte, 1988).

Acetylcholine caused this relaxation, independent of NOS and COX, and oubain inhibited the smooth muscle hyperpolarisation, suggesting the involvement of the Na-K ATPase. Following these studies, it was suggested that there was a diffusible factor,

produced by the endothelium, that was causing hyperpolarisation of the smooth muscle cell, and the term endothelial derived hyperpolarising factor (EDHF) was coined (Taylor & Weston, 1988). Further work in rabbit carotid artery found that the EDHF response involved an increase in intracellular Ca^{2+} levels within the endothelial cells (Chen et al., 1990). This supported the idea of EDHF as a diffusible factor as like NO, with an increase in Ca^{2+} from store release and extracellular Ca^{2+} entry triggering release of the EDHF from the endothelial cell where it could then diffuse to the VSM leading to relaxation. Work then began to identify this EDHF, much as NO had been found to be the endothelial derived relaxing factor a few years before.

1.7.2 Candidates for a diffusible EDHF

When EDHF was originally described it was believed to be a single diffusible factor which, like NO, is produced and released from the endothelium and then acts on thevascular smooth muscle to cause relaxation. This hypothesis now seems unlikely due to the large amount of research that has demonstrated a number of different substances to be involved rather than one particular factor (Feletou & Vanhoutte,

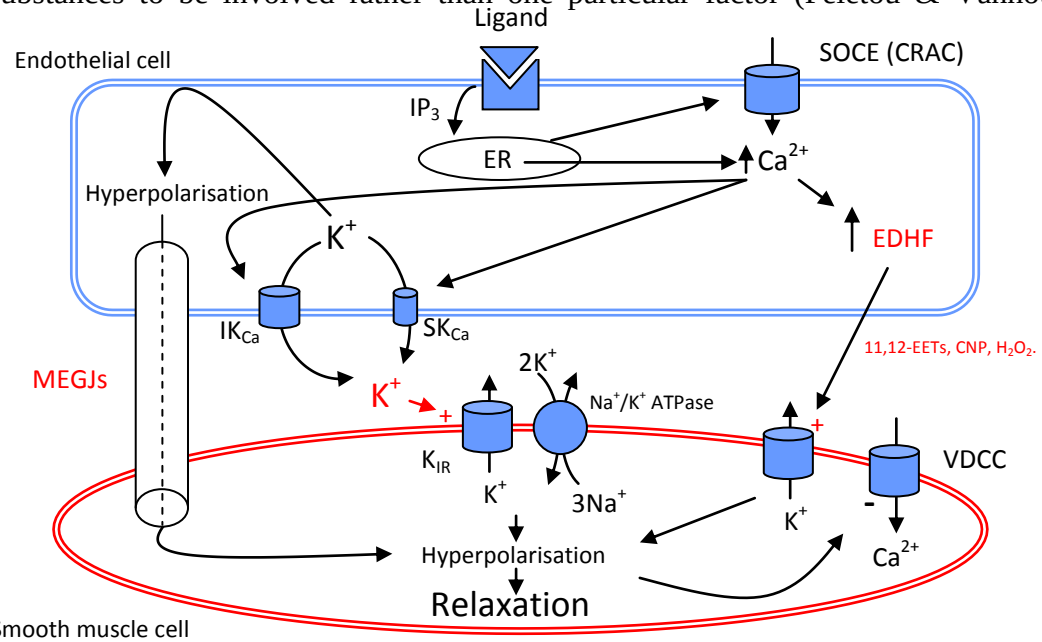

Figure 1.2. Scheme showing the different candidates for EDHF and the mechanisms leading to hyperpolarisation and relaxation of the smooth muscle.

2009) (see Fig. 1.2). There appear to be significant species and tissue differences between the pathways involved in causing hyperpolarisation of the vascular smooth muscle. These pathways have been suggested to act through various substances, including hydrogen peroxide (H_2O_2), the arachidonic acid metabolites epoxyeicosatrienoic (EET) acids, potassium ions (K^+), as well as through an electrical coupling mechanism in which myoendothelial gap junctions provide communication between the endothelial cells and smooth muscle (Bellien et al., 2008).

There are also thought to be a number of different substances that may be involved in modulating the EDHF response, for example through actions on channels which will affect the EHDF response (Dora et al., 2001). It is important to understand the exact mechanisms involved in the EDHF process so that its contribution in vascular regulation and possible therapeutic approaches can be explored (Yanga et al., 2007).

1.7.3 EETs

EETs are made from arachidonic acid by cytochrome P450 and have been demonstrated to be produced by endothelial cells (Campbell et al., 2010). They were proposed to be a possible diffusible EDHF as they are able to activate large calcium activated potassium (BK_{Ca}) channels on vascular smooth muscle leading to hyperpolarisation (Campbell et al., 1996). This was shown in bovine coronary arteries where EETs were produced in response to methacholine, a synthetic muscarinic receptor agonist. The addition of exogenous EETs could directly cause hyperpolarisation of the smooth muscle and relaxation. EETs were shown by patch clamping to increase the open-state probability of the BK_{Ca} channels on the coronary artery smooth muscle cells. The addition of arachidonic acid, which would increase

EETs production, also directly caused relaxation in the bovine coronary arteries and this response was inhibited by cytochrome P450 blockers. This study concluded that in the bovine coronary arteries investigated EETs were the primary EDHF.

Others have provided support for this view by showing the importance of cytochrome P450 in the EDHF response, implying that a product of the pathway could be the factor. For example, 17-ODYA blocked the response to acetylcholine recorded using intravital microscopy in hamster cremaster muscle (de Wit et al., 1999). However, more recently it has been proposed that mechanism by which EETs act could involve regulation of the Ca^{2+} influx into the endothelial cells; this would cause the opening of K^+ channels and subsequent hyperpolarisation. This was supported by evidence that elevated EETs cause the translocation to the cell membrane of a TRP channel in the endothelial cells (Fleming et al., 2007). The exact involvement of EETs is still to be clarified as in other tissues the arachidonic acid metabolite has also been shown to not be involved in the EDHF response (Baragatti et al., 2009, Campbell et al., 2010).

1.7.4 K^+

The EHDF response is characterised by a rise in endothelial $[Ca^{2+}]_i$ and the opening of Ca^{2+}-activated K^+ channels. As well as leading to hyperpolarisation of the endothelial cells, there is evidence that K^+ released from the endothelium may also act to directly cause hyperpolarisation of the VSMCs by opening K_{IR} channels and stimulating the electrogenic Na^+/ K^+ ATPase (Edwards & Weston 2004). The activation of K_{IR} and the ATPase can hyperpolarise the smooth muscle and lead to relaxation (Edwards et al., 1998). This study was carried out in small resistance vessels in rats in the hepatic

and mesenteric circulation, and showed that EDHF-induced relaxation was inhibited by Ba^{2+} and ouabain, which block K_{IR} and the Na^+/K^+ATPase, respectively. A small increase of extracellular $[K^+]$ was found to mimic the EDHF effect leading to hyperpolarisation of the smooth muscle. The response to K^+ was endothelium independent suggesting that the K^+ ions were affecting the smooth muscle cell directly. The authors proposed that in response to acetylcholine the IK_{Ca} and SK_{Ca} channels open leading to hyperpolarisation of the endothelial cell due to K^+ efflux. This will increase the K^+ concentration in the myoendothelial space leading to hyperpolarisation of the smooth muscle cell due to activation of the K_{IR} and Na^+/K^+ATPase. In accordance with this model, apamin and charybdotoxin abolished the relaxation to acetylcholine, but not affecting smooth muscle hyperpolarisation to the added K^+.

This scheme has been supported by work in the renal interlobar artery and porcine coronary arteries which demonstrated that the EDHF response corresponds to K^+ being released from the endothelium and then activating the Na^+/ K^+ ATPase (Beny & Schaad 2000; Bussemaker et al., 2002). The EDHF response to bradykinin was inhibited by ouabain, but not by barium or gap junction uncouplers. The involvement of K^+ was supported by the use of K^+ scavenger Kryptofix 2.2.2 which blocked the EDHF relaxation to bradykinin. However in the rat hepatic artery the activation of the Na^+/ K^+ ATPase and the K_{IR} channel by the ions was seen not to be involved as Ba^{2+} and ouabain had no effect (Andersson et al., 2000). This conclusion was also supported by electrophysiological studies which demonstrated that the hyperpolarisation of the vascular smooth muscle in the EDHF process was unlikely to be meditated by K^+ (Dong et al., 2000; Coleman et al., 2001). These discrepant results

may have been due to species and tissue differences, but they it is also possible that they arose from differences in the conditions used to study the EDHF response (see section 1.7.11). The species and tissue differences could be due to varying expression of channels and gap junctions which would alter the relative importance of the different pathways.

1.7.5 CNP

C-type natriuretic peptide (CNP) has also been proposed to be involved in the EHDF response. CNP is part of the family of vasoactive peptides that also includes ANP and BNP, which are involved in cardiovascular homeostasis. CNP has been shown to be found in high concentrations in vascular endothelial cells (Sandow & Tare, 2007). It is thought to activate NPR-C, one of the three natriuretic peptide receptors on the smooth muscle. In rat mesenteric resistance arteries, a selective NPR-C agonist mimicked the EDHF response and exogenous CNP elicited the same hyperpolarisation as acetylcholine (Chauhan et al., 2003). Following stimulation with acetylcholine there was an EDHF mediated relaxation that was blocked by barium and oubain suggesting K_{IR} and Na^+/K^+ATPase involvement. The relaxation was also blocked by 18α-GA a gap junction inhibitor. It is a possible diffusible mediator as it has been shown to lead to hyperpolarisation of the smooth muscle through activation of potassium channels (Simon et al., 2009). However subsequent work suggested that CNP does not play a significant physiological role in the EDHF process in a number of arteries. In mouse mesenteric arteries, for example, a CNP agonist did not cause relaxation and mice with NPR-C knocked out had the same EDHF response to wild types (Sandow & Tare 2007, see also Dora et al., 2008).

1.7.6 S-nitrosothiols

Additional substances may also have EDHF type activity. In porcine coronary microvessels S-nitrosothiols were seen to cause the activation of endothelial IK_{Ca} and SK_{Ca} channels, leading to hyperpolarisation of endothelial cells. These substances also have some direct effects on the vascular smooth muscle which could be involved in the EDHF response (Batenburg et al., 2004). The s-nitrosothiols may be able to activate smooth muscle Na^+/K^+ATPase via guanylate cyclase leading to relaxation. However, there is only limited research showing the involvement of s-nitrosothiols in the EDHF response (Batenburg et al., 2009).

1.7.7 Anandamide

Anandamide is an endogenous cannabinoid and has been suggested as having a role in the EDHF response, possibly through direct activation of BK_{Ca} channels (Griffith, 2004). However its role in the EDHF response has not been widely observed. For example, in rat cerebral arteries a blocker of anandamide had no effect on the EDHF response and inhibiting its uptake did not potentiate the response. This suggests that anandamide does not act as an EDHF in a wide range of tissues and may work down a different pathway (Dong et al., 2000).

1.7.8 H_2S

Hydrogen sulphide (H_2S) has been suggested as an EDHF as it could act as a diffusible gas like NO (Feletou & Vanhoutte, 2007). However though H_2S can cause relaxation it appears to work through a different pathway to that of EDHF. It has also been shown to be produced in smooth muscle rather than the endothelium suggesting that it is not EDHF (Baragatti et al., 2009).

1.7.9 NO and PGI_2

NO and PGI_2 are important mediators of endothelium dependent relaxation. They can directly cause hyperpolarisation but this is only weak. They also work through different pathways by acting directly on channels on smooth muscle with no involvement of the IK_{Ca} and SK_{Ca} channels which are characteristic of the EDHF response. The EDHF response has also been shown to be independent of these mediators by the use of L-NAME and indomethacin to block their production **(**Brakemeier et al., 2003**).**

1.7.10 MEGJ and the 'EDHF response' as a result of electrical coupling between the endothelium and VSMC

With a large amount of research failing to find a universal EDHF, the idea of the response as a process rather than a single diffusible factor has become more prominent (de Wit & Wolfle, 2007). Myoendothelial gap junctions (MEGJs) provide for the diffusion of current and small molecules between endothelial and smooth muscle cells.

Gap junctions are formed from the proteins called connexins. 12 of these subunits (6 from each cell) unite to form connexons which span both cell membranes at sites at which cells are closely apposed, with arrays of connexons forming gap junctions. Ions can flow through these channels, allowing changes in potential, and therefore in electrical excitation, to flow between cells. MEGJs spans intercellular gap between endothelial and adjacent VSMCs. Endothelial cells are also linked with each other via homocellular gap junctions, as well as by the heterocellular MEGJs with the underlying VSM cells (Levick, 2010). The vascular connexion subtypes are 37, 40, 43 and 45. The construction of gap junctions by these subtypes varies between species and tissues with differing formation in different size vessels (de Wit & Griffith, 2010).

Taking into account the existence of MEGJ, it was proposed (Chaytor et al., 1998; Chaytor et al., 2003) that following the hyperpolarisation of endothelial cells caused by the opening of K_{Ca} channels as a consequence of the action of agonists or shear stress, hyperpolarising current may spread to the VSMCs through the MEGJs (de Wit & Griffith, 2010). The tunica media of arterioles is only a few cells wide and MEGJ are abundant so there is good electrical coupling between these cells types in the resistance vasculature (Levick, 2010). This will then lead to smooth muscle cell hyperpolarisation and relaxation. In vitro in the vasculature of the hamster cheek, endothelial cells and smooth muscle were shown to be electrically coupled as their electrical responses were identical (Xia et al., 1995). The importance of communication between the cells in the EDHF process was demonstrated by the use of gap 27 which inhibits gap junction function. This was shown to inhibit EDHF

relaxations, indicating that gap junctions were underpinning the smooth muscle hyperpolarisations (Chaytor et al., 1998, Chaytor et al., 2003).

There is now extensive corroborating evidence for the involvement of MEGJs in the EDHF response. In rat mesenteric arteries, for example, angiotensin II reduced the EDHF response and part of this inhibition was due to decreased expression of connexins which form the gap junctions in the arterial wall (Dal-Ros et al., 2008). The involvement of MEGJs may also help explain some of the species and tissue differences that have been found with EDHF. This could also account in part for differences between results obtained in vivo and in vitro where there could be different regulation of the gap junctions (de Wit et al., 2008). The increased role of EDHF in smaller vessels may also be explained by MEGJ involvement as there would be greater communication between the endothelial cells and smooth muscle due to the smaller number of layers of smooth muscle. Studies on the localisation of K_{Ca} channels and MEGJs have shown differences in channel density which correlate with functional EDHF activity (Sandow et al., 2006). It has also been shown that SK_{Ca} channels may cause hyperpolarisation of the smooth muscle mainly through MEGJs while IK_{Ca} appear to act through the Na^+/K^+ ATPase, supporting a role for both MEGJs and K^+ in the EDHF response (Dora et al., 2008). Studies on an extract from a leaf used as a Chinese herbal remedy for hypertension have even shown that it acts through activation of the EDHF response involved K^+ channel activation and gap junctions (Jin et al., 2008). The use of a connexin 40 antibody which inhibited gap junction function demonstrated the importance of MEGJ communication in EDHF mediated dilatation (Mather et al., 2005). MEGJs not only help explain the EDHF response in physiological conditions but also pathological states as well. In diabetes

communication between cells has been found to be affected which could be responsible for the reduced EDHF response and the vascular problems associated with the disease (Figueroa & Duling, 2009).

The spread of the electrical current through gap junctions following endothelial cell hyperpolarisation has shown to be sufficient to cause hyperpolarisation and relaxation in the vascular smooth muscle (Sandow et al., 2002; de Wit et al., 2008). The localisation and density of MEGJs may help to explain some of the differences found in the EHDF response between tissues (Sandow & Hill, 2000). The electrical coupling also supports the hypothesis of EDHF being a process rather than a factor and may provide a general mechanism that underlies the hyperpolarisations (Griffith, 2004). It is therefore possible that ROS and other substances which have been proposed to be EDHFs may act on the processes that lead to the endothelial hyperpolarisation and the spread to the smooth muscle cell through the MEGJs, rather than themselves acting to hyperpolarise vascular smooth muscle.

1.7.11 Interaction between MEGJ and K^+ channels in EDHF response

Current work suggests that K^+ may be acting as an EDHF at low levels of VSMC excitation. K^+ released from the opening of SK_{Ca} and IK_{Ca} channels on the endothelium leads to the hyperpolarisation of the smooth muscle due to activation of the Na^+/K^+ATPase (Mather et al., 2005). At greater levels of VSMC excitation, the opening of BK_{Ca} channels on the VSMCs causes a large amount of K^+ efflux from these cells. This leads to an increased K^+ concentration in the myoendothelial space (the 'K^+ cloud') and this swamps any effect of K^+ released from the endothelium,

which therefore loses its ability to act as an EDHF (Edwards & Weston, 2004). However the relaxation to acetylcholine is still maintained by another mechanism, and there is evidence that the EDHF response at higher levels of VSMC stimulation is due to EC/VSM coupling via MEGJs. The gap junction inhibitor gap 27 blocked the smooth muscle hyperpolarisation only when the vessel was being stimulated by a higher concentration of phenylephrine (Richards et al., 2001). This suggests that K^+ acts as an EDHF under basal conditions, but when there is greater excitation, MEGJs become the dominant mechanism (Mather et al., 2005; Dora et al., 2008). These separate pathways working together under different conditions may to some extent explain the varying results found in different studies.

1.7.12 Hydrogen Peroxide as an EDHF

H_2O_2 is a ROS which is produced from superoxide either spontaneously or by the action of superoxide dismutase (SOD). H_2O_2 can be converted into the more reactive hydroxyl radical through the Fenton reaction (1) or be broken down by catalase. As described in this section, there is evidence that H_2O_2 can function as an EDHF in some vascular beds. The production of H_2O_2 and other ROS, and their effects on vascular cells, are described in more detail in subsequent sections of the Introduction.

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$

Matoba and co-workers originally reported that H_2O_2 caused an EDHF-like hyperpolarisation in small mesenteric arteries from the mouse (Matoba et al., 2000). In support of the concept that H_2O_2 was acting as an EDHF in these arteries, they reported that catalase inhibited the EDHF relaxation and hyperpolarisation. They

suggested that eNOS was a major source of the ROS, since eNOS KO mice had reduced EDHF responses to acetylcholine. In endothelium denuded arteries, exogenous H_2O_2 caused VSM relaxation and hyperpolarisation, suggesting it was working directly on the smooth muscle. Using peroxide sensitive fluorescent dyes and laser confocal microscopy they showed that H_2O_2 was produced in the endothelium following acetylcholine stimulation (Matoba et al., 2003). The group presented evidence that in human mesenteric arteries H_2O_2 could act as a primary EDHF, but also found that gap junctions are involved (Matoba et al., 2002). Others have also reported that endogenously produced H_2O_2 can act as an EHDF in vivo and is important in the regulation of coronary vascular tone (Yada et al., 2003). Shear stress has been shown to cause endothelial release of H_2O_2 and application of SOD, which should increase H_2O_2 levels, enhanced EDHF mediated relaxation in human mesenteric arteries, consistent with H_2O_2 being involved (Miura et al., 2003; Morikawa et al., 2004). In human coronary arterioles taken from patients undergoing cardiopulmonary bypass, H_2O_2 was shown to be involved in the EDHF response to shear stress (Liu et al 2011). In this study, 2 arterioles were cannulated and perfused in series. In response to shear stress applied to the upstream artery only, effluent from this vessel caused an iberiotoxin- and PEG-catalase-sensitive relaxation in the downstream endothelium denuded vessel. This suggests that H_2O_2 produced from the endothelium in response to shear stress was acting on the BK_{Ca} channels on the smooth muscle to cause relaxation. The opening of BK_{Ca} channels was confirmed using patch clamp studies on the smooth muscle cells.

These studies suggest that H_2O_2 is an EDHF, at least in some vascular beds. In porcine coronary arteries, H_2O_2 can cause relaxation through activation of BK_{Ca}

channels (Barlow & White, 1998), and it is possible that this could account for its hyperpolarising effect. However activation of the BK_{Ca} channel is not generally a characteristic of the EDHF response and it has been shown that although H_2O_2 can hyperpolarise and relax smooth muscle, in some arteries it seems to be distinct from EDHF itself because catalase did not block the EDHF response to substance P or bradykinin (Beny & von der Weid, 1991). It is also questionable whether the endothelium is able to produce concentrations of H_2O_2 which are sufficient to cause relaxation (Griffith, 2004).

In contrast to the proposal of Matoba's group that H_2O_2 is EDHF, there is now much evidence showing that H_2O_2 is involved in the EDHF response but is not a diffusible factor acting on the smooth muscle. For example, several studies found that exogenous H_2O_2 and catalase did not affect vascular smooth muscle (Hamilton et al., 2001; Gluais et al., 2005). Other studies have shown that while MEGJs are the main mechanism in the spread of hyperpolarisation to the smooth muscle, H_2O_2 is involved in the process (Chaytor et al., 2003). This was supported by the finding that in human gut mucosal arteries H_2O_2 is produced in response to acetylcholine and catalase inhibited the EDHF response, but that exogenous H_2O_2 did not cause relaxation of these arteries when they were denuded of endothelium (Hatoum et al., 2005). This supports the possibility that H_2O_2 acts in an autocrine or paracrine fashion on the endothelium itself to potentiate the EDHF response. Notably, Edwards and colleagues proposed in 2008 that H_2O_2 may enhance the EDHF response by increasing intracellular calcium in the endothelial cell which causes hyperpolarisation. This was based on experiments which showed that H_2O_2 and thimerasol, a thiol oxidant, enhanced endothelial Ca^{2+} release and endothelium-dependent relaxation caused by

the SERCA inhibitor CPA. The relaxation to CPA was diminished by catalase and also by the connexion blocking peptide ^{43}Gap 26. These results suggested that H_2O_2 might be promoting endothelial cell Ca^{2+} release through the IP_3 receptor which would cause hyperpolarisation that spreads to the vascular smooth muscle via MEGJs, or possibly could cause the release of other factors (Edwards et al., 2008). This hypothesis fits with the central role of MEGJs which have been shown in many studies. H_2O_2 may also act directly on K_{Ca} channels through thiol group modification (Cai & Sauve 1997), thus promoting endothelial cell hyperpolarisation. H_2O_2 can signal through a number of mechanisms including kinases and calcium rises in endothelial cells in response to H_2O_2 have been demonstrated (Hu et al., 1998; Jornot et al., 1999; Sobey & Miller 2005). Phospholipase C is a possible pathway that H_2O_2 activates and it seems to be the main ROS involved rather than superoxide or the hydroxyl radical (Volk et al., 1997; You et al., 2002). However, while many papers have demonstrated the involvement of H_2O_2 in the EDHF response, research into the actual mechanisms and the possible involvement of other ROS is limited. It is therefore important to understand how H_2O_2 and ROS are involved in the EDHF response, and if they are potentiating the rise in intracellular calcium in the endothelial cells what pathways are involved.

1.8 ROS

ROS are molecules that are chemically reactive and contain oxygen. These include free radicals which have one or more unpaired electron and also H_2O_2, which does not have an unpaired electron. There are many sources of ROS and the different types of ROS such as superoxide ($O_2^{\cdot-}$), H_2O_2 and the hydroxyl radical ($^{\cdot}OH$), can have a wide

range of varying effects (see Fig. 1.3) (Li & Shah, 2004). Superoxide is produced by a number of oxidases as part of normal cellular metabolism. This can then be converted to H_2O_2 by superoxide dismutase. In the presence of iron the Fenton reaction can take place which produces the hydroxyl radical, or H_2O_2 can be broken down by catalase to produce water. Superoxide can also react with NO to produce the ROS peroxynitrite (Faraci et al., 2006).

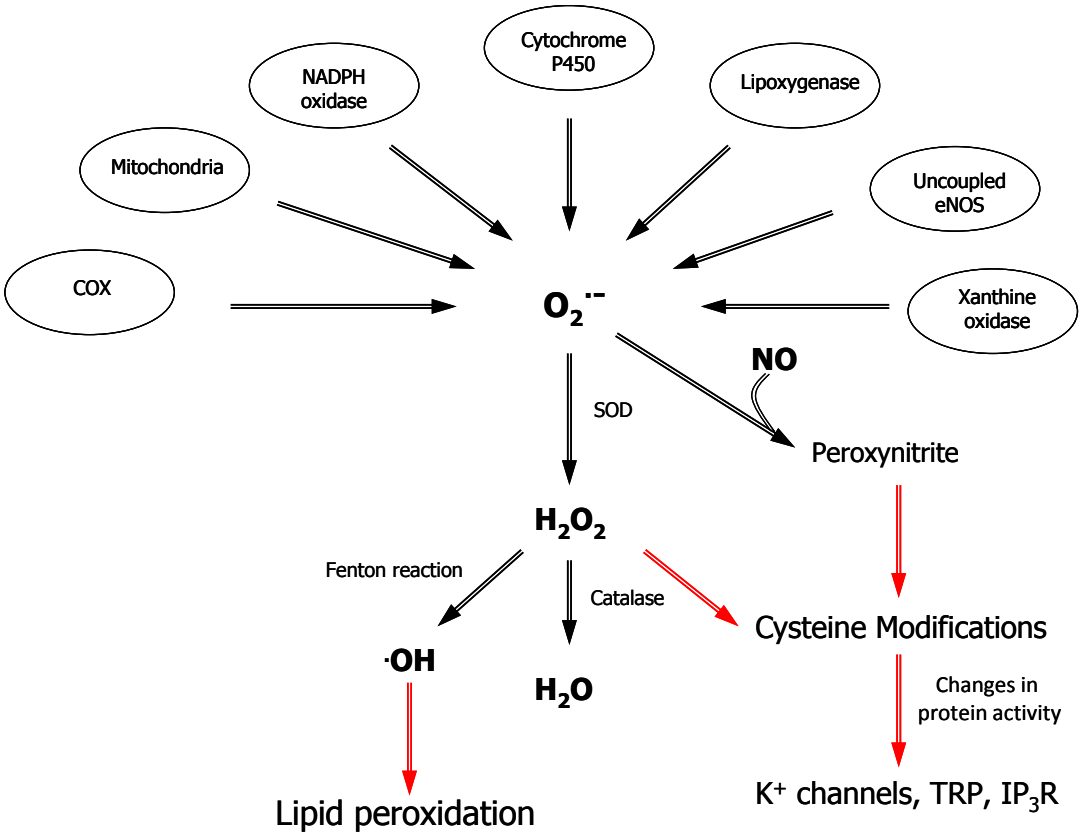

Figure 1.3. Scheme showing the main pathways for ROS production and their downstream targets.

There are many enzymes and processes involved in ROS production including NADPH oxidase, xanthine oxidase, NOS, COX and cytochrome P450. In endothelial cells the metabolism of arachidonic acid by cytochrome P450 enzymes is an important source of ROS generation (Fleming et al., 2001). Another source of ROS is also from aerobic respiration in the mitochondria (Husain et al., 2008). For example,

following ischemia-reperfusion the tissue injury seems to be partly due to increased ROS production by xanthine oxidase and mitochondrial complexes (Baudry et al., 2008).

Oxidative stress due to overproduction of ROS is thought to be involved in cardiovascular disease (Rojas et al., 2006). However there is now growing evidence that at lower concentrations ROS are important in physiological signalling, and the cellular redox state is important in regulating cellular function (Janssen-Heininger et al., 2008). Changes in redox balance and in particular the effects on NO bioavailability can affect vascular function, but at normal levels ROS are being increasingly proposed to play an important role in signal transduction (Hare et al., 2004), an example of this being the concept that H_2O_2 is EDHF. This may partly explain why the use of anti-oxidants therapeutically has not had the beneficial effects that were hoped for (Bjelakovic et al., 2008).

1.8.1 Sources of ROS

1.8.2 NADPH oxidase

NADPH oxidases (Nox), of which there are 5 types, are an important source of ROS, and the different Nox proteins could be targeted pharmacologically for therapeutic purposes (Brandes & Schroder 2008). The enzyme transports electrons across the plasma membrane and reduces oxygen, generating superoxide and other downstream ROS. The different enzymes can be modulated by other factors such as the GTP binding protein Rac 1 which in several types of Nox can cause an increase in

superoxide generation (Hassanain et al., 2007). Nox, originally described in phagocytes where it has a role in host defence, now is thought to have roles in cell signalling and gene expression.

The Nox subtypes are expressed in different cells and have different subunits and regulatory proteins. Nox1 is found to be expressed in the endothelium and VSMC, as well as other tissues and organs including the colon. It is activated by Rac and PKC can increase its expression. Nox2 is also expressed in endothelial cells and VSMC, and was the originally described enzyme that was found in phagocytes. Nox2 expression is increased by angiotensin II. Nox3 seems to not be important in the vasculature but is present in the inner ear. Nox4 is found in endothelial cells and VSM as well as fibroblasts. It is thought to be constitutively active so does not require cell stimulation but it can be unregulated by angiotensin II or TNFα and increased expression occurs following shear stress and hypoxia. Nox5 is found in testis and lymphoid tissue as well as VSM and endothelial cells. The DUOX1 and 2 enzymes are also Nox subtypes and are found in glands including the thyroid and testis as well as the epithelial cells in the airway (Bedard & Krause, 2007).

Nox2 is the best described Nox subtype. It is made up of 6 transmembrane domains with the COOH terminus and NH_2 terminus facing the cytoplasm. It consists of the Gp91phox catalytic subunit (also called Nox2) which is bound to p22phox in the cell membrane. The active Nox associates with the p40phox, p47phox and p67phox regulatory cytosolic subunits which form a complex (Li & Shah, 2004). All the subtypes have a conserved NADPH binding site at the COOH terminus. Activation of Nox2 requires translocation of the cytosolic subunits to the Nox2-p22phox complex

in the cell membrane. This occurs by the phosphorylation of p47phox which organises the translocation of the other cytosolic subunits. This causes a conformational change which allows the direct interaction of p67phox with p22phox. The GTP binding proteins Rac1 and Rac2 can then interact with Nox-2 via the activator subunit p67phox (Bedard et Krause. 2007). The p47phox acts as an organiser subunit and PKC is thought to be one of the kinases that can activate it via phosphorylation (Fontayne et al., 2002). The activated complex can then generate superoxide by the transfer of an electron from NADPH in the cytosol to oxygen in the extracellular or luminal space. Nox1 and Nox3 also require the p22phox subunit and Rac for activation. Nox4 requires p22phox but is constitutively active. Nox5 and DUOX1 and 2 are activated by Ca^{2+} and do not require the translocation of subunits for activation. In the endothelial cell the subunits may be intracellular rather than plasma membrane bound and there may be a constant low level of superoxide production by Nox4 (Li & Shah. 2004). Unlike other sources of ROS, the primary role of the Nox enzymes appears to be the production of superoxide rather than it being a byproduct, so it may be important in cell signalling that involves ROS (Brandes & Schroder, 2008).

1.8.3 Cytochrome P450

Cytochrome P450s (CYP450) are a family of heme containing enzymes. In mammals they are membrane bound, in particular on the endoplasmic reticulum and mitochondria. They are involved in the metabolism of a number of substances including cholesterol, vitamins, steroids and exogenous compounds such as drugs like ethanol or the anticoagulant warfarin (Myasiedova et al., 2008). The CYP450 enzymes are involved in oxidation, peroxidation, hydroxylation and reduction of

different compounds. The enzymes are found in most cell types and are especially important in metabolism in the liver. CYP450 is also expressed in endothelial cells and VSMC. CYP450 enzymes can produce ROS, particularly members of the CYP2 family (Fisslthaler et al., 2000). During metabolism superoxide can be produced by the electron transfer from the heme iron to a oxygen molecule in an NADPH dependent reaction. This has been shown to occur during the metabolism of ethanol by CYP2E1 in hepatocytes (Myasiedova et al., 2008). CYP450 are expressed in endothelial cells and could be involved in vascular regulation. CYP2C9 has been shown to be present in porcine coronary arteries and human coronary arteries where it was shown to be a significant source of ROS (Fleming et al., 2001). CYP2C9 can metabolise arachidonic acid to produce a number of products including ROS (Li & Shah, 2004). This suggests that CYP450 could be important in vascular regulation through the production of ROS involved in cell signalling.

1.8.4 eNOS

NOS is the enzyme involved in NO production. eNOS is the type of NOS found in endothelial cells; it is Ca^{2+} dependent and generates NO and L-citrulline by the oxidation of L-arginine which involves the reduction of molecular oxygen. This happens by eNOS shuttling the electrons from the reductase domain to the oxidase domain (Rabelink et al., 2006). Tetrahydrobiopterin (BH_4) is a cofactor that couples the reduction of the molecular oxygen to the L-arginine oxidation. BH_4 bioavailability is reduced when there is increased oxidative stress as it is susceptible to oxidative degradation into BH_2. If BH_4 levels are reduced, eNOS can become uncoupled leading to the generation of superoxide rather than NO (Landmesser et al., 2003).

Oxidative stress therefore is amplified as it leads to further ROS production by uncoupling eNOS. If there are low levels of L-arginine, the eNOS complex can dissociate leading to the production of H_2O_2. This ROS production by uncoupled eNOS could be important in redox signalling. It has been suggested that this could be involved in the development of atherosclerosis, as well as a physiological role in host defence (Rabelink et al., 2006).

1.8.5 Mitochondria

Mitochondria can be a major source of ROS in cells. During aerobic metabolism, the mitochondrial respiratory chain produces superoxide (Husain et al., 2008). Substrates such as fatty acids are broken down to produce the electron donors NADH and $FADH_2$. Electrons from these donors are then shuttled into the electron transport chain. The electron transport chain transfers electrons through different complexes down a redox gradient. This provides energy which is used to pump protons from the mitochondrial matrix to the intermembrane space. The energy from the proton gradient is then used by ATP synthase in complex V to generate ATP (Burwell & Brookes, 2008). Molecular oxygen is the final electron acceptor for cytochrome C oxidase at the terminal end of the respiratory chain leading to the producing of water. However 1-4% of the oxygen may be incompletely reduced resulting in the production of superoxide, mainly at complex I and III (Li & Shah, 2004). There is thought to be increased production of ROS by mitochondria during certain diseases such as diabetes and mitochondrial dysfunction has been implicated in ischemia-reperfusion injury (Burwell & Brookes. 2008).

1.8.6 X-XO

Xanthine oxidase is an oxidoreductase which is important in ROS production (Baudry et al., 2008). Xanthine oxidase is generated by post-translational modification of xanthine dehydrogenase which is constitutively expressed *in vivo* in most cells. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and xanthine urate (Kozlovski et al., 2006). This involves the reduction of molecular oxygen which acts as an electron acceptor. This generates superoxide and is also a major source of H_2O_2 which is produced from the oxidation of hypoxanthine and xanthine (Li & Shah, 2004). Conversion of xanthine dehydrogenase to xanthine oxidase involves reversible thiol modification of sulfhydryl residues which is increased during hypoxia and by inflammatory mediators like TNFα. Xanthine oxidase has been shown to be expressed at high levels on the luminal surface of the vascular endothelium so could be a major source of ROS generation (Li & Shah, 2004).

1.8.7 H_2O_2 and cell signalling

H_2O_2 is a ROS that is produced from superoxide by superoxide dismutase (SOD). CuZnSOD is present in almost all cells and is located in the cytosol while MnSOD is mainly found in the mitochondria (Halliwell and Gutteridge, 2007). H_2O_2 can then be broken down by catalase into water, or by the Fenton reaction converted into the hydroxyl radical. H_2O_2 itself is not a free radical as it does not have an unpaired electron. This means it is less reactive and this increased stability means that it is well suited as a potential intracellular signalling molecule, rather than causing oxidative damage. Its stability allows it to diffuse through aquaporins or directly though the cell

membrane where it could act on downstream targets (Janssen-Heininger et al., 2008). Superoxide on the other hand is also not very reactive but is quite unstable and its negative charge makes it poorly diffusible through cell membranes. The hydroxyl radical is highly reactive with a very short half life so would lead to less specific oxidation and would not be able to diffuse to target proteins. In most tissues it is thought that H_2O_2 is produced continually, with normal concentrations thought to be around 10-100nM range, though this could be higher in microdomains where there can be a localised concentration. Microdomains are a region in the cytoplasm that have distinct conditions or function. This can form around channels or enzymes which lead to a local increase in concentration which can be higher than in the rest of the cell allowing for specific signalling within this region. Microdomains can form in caveolae, which are lipid rafts that form at invaginations of the plasma membrane and allow for specific singal transduction due to the localisation of channels such as gap junctions (D'Autreaux et al., 2007). Though it is poorly reactive in general, it can cause oxidative modifications of thiol groups on cysteine residues, which can alter protein function. The cysteine residue can cycle between different stable forms depending on the redox state and the residues have different susceptibility to undergoing redox modification. This selective reactivity makes H_2O_2 ideal for signalling and the reversible modifications of the cysteine residues allow for regulation (Forman et al., 2004).

H_2O_2 can cause specific oxidation of target cysteine residues within proteins which causes production of sulfenic acid intermediates. These intermediates are then glutathionylated by the addition of the peptide glutathione. H_2O_2 can also cause the formation of disulfide bonds between two cysteine residues forming cysteine (Gupte

et al., 2008). This covalent modification leads to a change in the tertiary structure which can alter the function of the protein. These redox modifications can be specific because different cysteine residues have differing levels of reactivity. The ionisation state of cysteine is important with residues with a low pKa in the thiolate form (S^-) more available for oxidation. The groups in the thiolate form are more nucleophilic so are more able to donate an electron and be oxidised (DiChiara et al., 1997). The cysteine residues themselves can serve in redox regulation by interacting with zinc as this metal's binding can lower the pKa of the thiol thereby modifying its reactivity. The reactivity of the residues is only determined by their localisation within the protein, with neighbouring positively charged residues stabilising the bonds by raising the pKa (D'Autreaux et al., 2007). This leads to the reaction rates with the thiol groups varying which confers specificity and selectivity. The specificity of ROS signalling will also be conveyed by the regulation of ROS production, and by ROS levels being locally compartmentalised allowing for tight modulation of ROS levels. The redox state will also be determined by the balance with antioxidant pathways (Winterbourn & Hampton, 2008).

These modifications have been shown on a range of proteins including enzymes such as tyrosine kinase and channels (Janssen-Heininger et al., 2008). ROS and in particular H_2O_2 could be therefore important in specific and reversible cell signalling through direct modification of protein function.

1.9 Effects of ROS

As described above, ROS can have a role in cell signalling via redox modification of proteins which can alter their function. This could involve the activation or inhibition

of transcription factors, membrane channels or enzymes (Winterbourn & Hampton, 2008). As described, the oxidation and reduction of thiol groups of reactive cysteine residues of proteins is thought to be a major mechanism of regulating different pathways. The role of redox modification in the control of vascular tone, and in particular mechanisms involved in the EDHF mediated relaxation is discussed below.

1.9.1 IP_3R

The IP_3 receptor is activated by the release of IP_3 which is produced with DAG by hydrolysis of PIP_2 by PLC. The negatively charged molecule diffuses to the sarcoplasmic reticulum where it binds to the IP_3 receptor causing the opening and release of Ca^{2+} from the internal stores. This increases the intracellular Ca^{2+} concentration which has downstream effects as previously described (Hidalgo et al., 2008). Thiol groups on the IP_3 receptor could be modified by ROS which can increase the sensitivity of the receptor to IP_3. This happens by conformational change of the channel which makes it more sensitive to IP_3 binding.

In human aortic endothelial cells, NOX derived H_2O_2 increased the sensitivity of stores to IP_3. Pre-incubation with concentrations of 100µM H_2O_2 were used to potentiate the response to acetylcholine by increasing the store release of Ca^{2+} leading to increased relaxation (De Wit 2010). In rabbit iliac arteries, exogenous H_2O_2 was found to enhance the EDHF response elicited by the SERCA inhibitor cyclopiazonic acid (CPA) (Edwards et al., 2008). This effect was mimicked by using the sulfhydryl reagent thimerosal which can oxidise the thiol group on the IP_3 receptor. This enhanced release of Ca^{2+} from endothelial stores potentiates the EDHF response by

increasing endothelial K_{Ca} channel activity. The response to H_2O_2 was sensitive to K_{Ca} channel inhibition showing that it is acting on the endothelial cells to enhance to EDHF response, rather than working directly on the smooth muscle. The response was also blocked by catalase and potentiated by a catalase inhibitor, further supporting the role for H_2O_2.

In human aortic endothelial cells, the addition of exogenous H_2O_2 itself has been shown to cause transient increases in $[Ca^{2+}]$. This was shown to be from store release of Ca^{2+} as removal of Ca^{2+} from the buffer solution did not block the increase whereas prior depletion with thapsigargin did abolish the rise (Hu et al., 2008). This suggests that H_2O_2 can directly open the Ca^{2+} store channels.

The EDHF response to histamine was shown to involve an increase in ROS production, demonstrated by an increase fluorescence of DCF in human aortic endothelial cells (Hu et al., 2002). 10μM H_2O_2 had no effect on $[Ca^{2+}]$ in the endothelial cells but the Ca^{2+} increase to histamine was enhanced by pre-incubation with H_2O_2. This response was not blocked by removal of extracellular Ca^{2+} so it was suggested that H_2O_2 was increasing the sensitivity of the endoplasmic reticulum to IP_3.

These observations suggest that H_2O_2, rather than being an EDHF factor itself, could potentiate the response by increasing Ca^{2+} release from endothelial stores (Edwards et al, 2008).

1.9.2 TRPM2

ROS may be able to directly modulate the activity of channels, including the TRP family of cation channels. For example, TRPC2 is widely expressed and exogenous H_2O_2 could directly open the cation channel. Increased levels of arachidonic acid which could increase ROS production also increased channel activity (Hara et al., 2002). The activation of the TRPC2 channel by H_2O_2 appears to be by direct modification, rather than by affecting regulators of the channel such as ADP ribose. This was shown in a patch clamp study by the addition of H_2O_2 increasing the current through the TRPC2 channel (Wehange et al., 2002). TRPM2 in particular has been demonstrated to be sensitive to redox modification and could have a role in regulation of vascular tone (Yao 2005; Kwan 2007). H_2O_2 was shown to stimulate ADP ribose formation which leads to the opening of the TRPM2 channel (Kraft 2004). This could be important for vascular regulation as TRPM2 expression was shown in endothelial cells and could cause an increase in Ca^{2+} entry in response to H_2O_2 (Hecquet et al., 2008). This effect was blocked by the use of a TRPM2 antibody using a model with TRPM2 overexpressed which enhanced the Ca^{2+} entry in response to H_2O_2. The redox sensitivity of the TRPM2 channel could be involved in the EDHF response as Ca^{2+} is an important part of the signalling pathway (Naziroglu et al., 2008).

1.9.3 K^+ channels

K^+ channel activation, in particular of K_{Ca} channels, is an important part of the EDHF response. ROS may be able to directly modify the K^+ channels to enhance the EDHF response. This could occur via oxidation of the cysteine residue near the Ca^{2+} sensor

in the K_{Ca} channels which could increase their sensitivity. ROS have been shown to directly affect the activation of K_{Ca} channels, K_{ATP} and K_V channels (Gutterman et al., 2005).

In human coronary arterioles, H_2O_2 was shown to be produced in response to shear stress (Liu et al 2011). The H_2O_2 appeared to act on the smooth muscle via BK_{Ca} channel activation as its vasodilating effect was sensitive to iberiotoxin. Patch clamp studies showed that exogenous H_2O_2 increased the open probability of the channel. However, the effect of ROS may be concentration dependent, or vary between tissues as in porcine renal arteries, H_2O_2 was found to cause a concentration dependent inhibition of BK_{Ca} channel activity (Brakemeier et al., 2003). This demonstrates that redox modification can have differing effects on a channel, possibly due to an alteration in the sensitivity of the thiol group to the ROS or other regulatory factors. This could be affected by the concentration of ROS, as well as the tissue and location (Gutterman et al., 2005).

In bovine aortic endothelial cells patch clamping was used to investigate the effect of oxidative reagents on K_{Ca} channel activity (Cai et al., 1997). The use of oxidative reagents such as thimerosal reduced the K_{Ca} channel activity. This was also shown by the addition of H_2O_2 also suppressing channel activity, which could be restored by using reducing agents. However in rat cerebral arteries, relaxation was found to be blocked by catalase and by BK_{Ca} channel inhibitors (Sobey et al., 1998). This suggests that H_2O_2 was working through activation of the BK_{Ca} channels to cause relaxation. This variation was shown within one study in rat skeletal muscle arterioles (Samora et al., 2008). In juvenile rats, the relaxation to acetylcholine was blocked by

catalase and the K^+ channel inhibitors TEA and glibenclamide. There was also an increase in DCF fluorescence in response to acetylcholine signalling an increase in ROS production. This relaxation could be repeated by the addition of exogenous H_2O_2, also working through the K_{Ca} and K_{ATP} channels. However, in younger weanling rats, catalase did not block the relaxation to acetylcholine, and exogenous H_2O_2 had no effect. This suggests that H_2O_2 in juvenile rats caused relaxation through the opening of K^+ channels, but in weanling rats a different mechanism was involved. These differences could be due to an alteration in the channel structure which changes the sensitivity of the thiol groups to redox modification.

Studies of BK_{Ca} channels on human embryonic kidney cells characterised the effect of redox reagents on channel activity (DiChiara et al., 1997). The application of a reducing reagent increased channel activation whereas H_2O_2 reduced the channel open-probability. This shows that as well as being modulated by the intracellular Ca^{2+} concentration, BK_{Ca} channel activity can be regulated by redox modification. The variability was demonstrated in electrophysiology studies on endothelial cells with low H_2O_2 concentrations inhibiting K_{IR} currents, whereas a higher H_2O_2 concentration increased the outward K_{Ca} current (Bychkov et al., 1999). The studies suggest that ROS, in particular H_2O_2 can directly modify K^+ channel activity, and this varies between tissues and can be concentration dependent.

1.9.4 Lipid peroxidation

There is increasing evidence for the role of ROS in cell signalling, and the modification of thiol groups allows for a specific and reversible pathway. However, ROS may also be able to alter function by direct lipid peroxidation which will affect

the structure and function of the cell (Khatchadourian et al., 2009). Oxidised lipids can affect signalling pathways and protein function. The hydroxyl radical is the most active species in lipid peroxidation as it is very reactive (Smith et al., 1994). The hydroxyl radical is formed via the Fenton reaction from H_2O_2. This requires Fe^{2+} to be present which catalyses the reaction to form the hydroxyl radical, and the reaction can be stopped by using iron chelators (Halliwell & Gutteridge, 2007). Lipid peroxidation occurs as ROS react with arachidonic acid in the cell membrane, producing an alkyl radical which can then react with neighbouring arachidonic acid molecules forming further radicals and lipid hydroperoxide. This cycle continues and can produce toxic aldehydes which can react with cysteine residues on proteins (Hall et al., 2010). The role of this process in cell signalling is less clear as lipid peroxidation is not specific or easily regulated like thiol modification.

Lipid peroxidation has primarily been implicated in oxidative damage, such as following trauma. Using the lipid soluble antioxidant vitamin E, which will scavenge ROS in the lipid layer, it has been shown that oxidative damage following trauma can be reduced in the brain (Mabile 1995).

1.10 EDHF in humans

1.10.1 Role in pathology and disease

Understanding the process by which EDHF causes relaxation is vital as it is thought to be important in normal physiological functioning and pathological states of the vascular system. EDHF seems to be of particular significance in the smaller vessels

and the microcirculation. NO is the primary relaxing factor in large conducting arteries, whereas in smaller vessels EDHF seems to be the dominant pathway (Garland et al., 1995). This could be partly due to lower expression of eNOS in the smaller arterioles (Shimokawa et al., 1996). As these are the resistance vessels, EDHF may well have a significant role in the control of blood pressure. EDHF also seems to be important in pathological states, and is thought to be upregulated in some conditions and reduced in others (Bellien et al., 2008). Significantly, when NO is suppressed, EDHF action has been shown to be increased, which may mean it can partially compensate for the loss in NO regulation. EDHF therefore may play a protective role through maintaining blood flow and organ perfusion (Bryan et al., 2005). EDHF and the mechanisms underlying the relaxation may therefore be a potential therapeutic target. Drugs that selectively activate the IK_{Ca} or SK_{Ca} channels could have potential benefits, particularly in diseases such as diabetes and atherosclerosis when the NO response is reduced (Feletou & Vanhoutte, 2004). The EDHF response was also found to be impaired in rat models of diabetes (Fitzgerald et al., 2005), and this could contribute to the poor vascular condition associated with this disease, particularly in the periphery. There appear to be species differences as in a mouse model of diabetes EDHF response was enhanced and in type 2 diabetes it was able to compensate for the diminished NO action (Fitzgerald et al., 2005; Park et al., 2008). The EDHF response has also been found to be reduced in pre-eclampsia, with this being particularly important due to the its role regulation of blood pressure by resistance arteries (Luksha et al., 2008). Due to EDHF's apparent involvement in pathologies and its potential as a therapeutic target it is important that the mechanisms are identified and the process understood.

1.11 Research flaws

There has been a significant amount of research looking at the identity of EDHF and much of it has resulted in conflicting observations and proposals. Though there will inevitably be variation in results due to species and tissues differences, part of this may be due to problems with the experimental design or unsafe conclusions being derived from the work. A good example of this is the use of ouabain which has been widely employed to show the involvement of the Na^+/K^+ ATPase, with the inhibition of relaxation by ouabain being used to suggest the importance of the Na^+/K^+ ATPase. However the inhibition of the Na^+/K^+ ATPase itself will lead to a rise in intracellular $[Na^+]$. This will reduce the activity of the Na^+/Ca^{2+} exchanger leading to an increase in cytosolic $[Ca^{2+}]$ which itself will inhibit relaxation. This means that the ouabain itself is directly inhibiting relaxation as it causes a calcium rise in the smooth muscle. Therefore drawing conclusions about the role of the Na^+/K^+ ATPase by examining the effects of ouabain on tension is difficult, unless membrane potential has also been recorded (Sandow et al., 2004).

The use of drugs which have a wide range of actions and drugs which cannot pass into cells also must be recognised. For example Volk's group used an iron chelator which does not readily move into cells so its lack of effect does not necessarily show intracellular hydroxyl radicals are not involved (Volk et al., 1997). A major flaw with much of the work with ROS and H_2O_2 is the use of high exogenous concentrations. ROS at higher concentrations will affect the redox balance of cells so could have a wide range of varying effects. H_2O2 can have differing effects depending on the concentration used as well so it is difficult to draw conclusions about the mechanisms

when high concentrations are used which may not be physiologically relevant (Forman, 2007).

1.12 Summary and aims

Following the identification of nitric oxide (NO) as the main endothelium derived relaxing factor (EDRF) other important factors have been recognised. This includes the finding in canine coronary arteries that there is an endothelium dependent hyperpolarisation which leads to relaxation (Feletou & Vanhoutte 1988). The mechanism behind the hyperpolarisation was found to be independent of NO and prostacyclin and it was termed endothelium derived hyperpolarizing factor. Despite its characterisation over 20 years ago the identity of EDHF is still controversial. One suggestion is that that reactive oxygen species (ROS) are involved. ROS have often been seen as having a damaging effect on vascular function through oxidative stress, but it now seems that they play important physiological roles in cellular signalling (Faraci et al., 2006).

The aim of my PhD project was to investigate the part played by ROS in the EDHF signalling pathway. This would lead to a better characterisation of the mechanisms involved in EDHF-induced relaxation, and provide an improved understanding of the physiological role of ROS. A more complete picture of the role of ROS in the regulation of the vasculature might also help explain findings from clinical trials that dietary anti-oxidants have no benefit in reducing cardiovascular disease (Bjelakovic et al., 2008).

Taking into the account evidence supporting an important but indirect role of H_2O_2 in the EDHF response, I hypothesised that endothelium- or smooth muscle derived H_2O_2 acts in an autocrine or paracrine manner on the endothelium to promote the rise in intracellular calcium in response to a stimulus such as acetylcholine. This would lead to a greater opening of K_{Ca} channels, and more of a rise in extracellular $[K^+]$ and endothelial cell hyperpolarisation which spreads to the smooth muscle through MEGJs. H_2O_2 would therefore potentiate the EDHF response rather than being the factor itself. I attempted to prove this hypothesis by simultaneously measuring the vascular diameter and endothelial cell Ca^{2+} concentration in arterioles of the rat cremaster circulation.

Chapter 2: General Methods

2.1 Introduction to Methods

Investigations into the EDHF response look at the resistance vessels of the microcirculation where this pathway is most prominent. The cremaster muscle, the thin skeletal muscle layer surrounding the testes, is an ideal tissue to study the microcirculation as the vessels can be visualised in situ using intravital microscopy (Vicaut & Stucker, 1990). The study of the rat cremasteric circulation has been used for nearly 40 years and allows the direct measurement of changes in the diameter of the blood vessels (Baez, 1973). By using calcium indicators this technique also allows for the simultaneous measurement of changes in endothelial calcium, a vital step in the EDHF response. Intravital microscopy also minimises damage to the vessels being studied as they remain in situ within the cremaster muscle and allows the study of smaller vessels that could not be easily dissected. Using in situ vessels allows greater control of variables unlike the in vivo model and removes affects of anaesthesia on the response (Majino et al., 1967). In the present study intravital microscopy was used to characterise the EDHF response in arteries in the cremasteric circulation and the role of ROS in this relaxation pathway.

2.1.1 Intravital microscopy

Intravital microscopy consists of a microscope attached to a camera which allows the direct visualisation of the blood vessels in situ. The tissue was prepared by setting up isolated perfusion of the cremasteric microcirculation and the exposed cremaster muscle was mounted on a viewing stage below the microscope.

2.1.2 Animal preparation

4-6 week old male Wistar rats weighing 80-100g were killed in accordance with Schedule 1 guidelines of the Home Office license. The procedure involved exposure to an increasing concentration of carbon dioxide gas followed by cervical dislocation. The surgical procedure to prepare the tissue was then started immediately to avoid blood clotting and tissue damage. The abdomen from the xiphisternum to the scrotum was shaved of fur with an electric razor to allow easier manipulation during dissection and to avoid hair affecting visualisation under the microscope. The animal was then secured on a clear plastic stage in the supine position ready for the surgical procedure.

2.1.3 Surgical procedure

A midline incision was made along the abdomen of the rat from the xiphisternum to the pubic symphysis and the skin and muscle layer were retracted to expose the underlying organs. The intestines were moved to one side to expose the abdominal aorta and inferior vena cava. The aorta and surrounding vessels were cleaned using blunt dissection of connective tissue and fat to allow for easier manipulation. A microscope (Zeiss x4) was used to aid careful dissection. The inferior vena cava was tied off with thread at a level above the bifurcation into the common iliac veins and below that an incision was made to allow drainage of the blood from the lower extremities. An incision was made into the abdominal aorta and a 0.61mm fine bore polythene cannula was inserted down to the bifurcation into the common iliac arteries. The cannula was secured with thread and the left common iliac artery and the right femoral and internal iliac arteries were ligated. This ensured perfusion was only to the

right external iliac artery which leads to the inferior epigastric artery and then the cremasteric artery which supplies the cremaster muscle. The cremaster was then perfused through the cannula with St. Thomas' cardioplegic solution with heparin (30U/ml) to remove blood and to suspend cellular function. The anticoagulant heparin was included to prevent thrombus formation within the cremasteric circulation while the blood was being drained. The circulation was flushed through with the solution until the blood was fully drained. The non-toxic Evan's Blue dye (1% Evan's Blue 1:5 Albumin) could be used to ensure good perfusion as it is easily visualised so filling of vessels confirmed vascular integrity.

2.1.4 Cremaster preparation

The cremaster muscle is the continuation of the internal oblique muscle which forms the muscle layer surrounding the testes. In young rats the muscle is thin so the vessels can be easily visualised using a microscope. In rats younger than 4 weeks the testes may not have descended while in older rats the muscle and connective tissue layers that the vessels run through are thicker so the arteries are more difficult to visualise.

The cremaster muscle was prepared by a midline incision along the scrotum to expose the right testes. Skin and connective tissue were carefully removed from the underlying cremaster muscle using careful blunt dissection to reduce tissue damage. The muscle was then cut longitudinally along the anterior surface from the apex to the

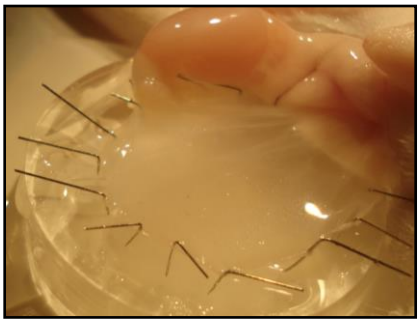

Figure. 2.1. Exposed cremaster muscle pinned to Sylgard

inguinal canal using microsurgical scissors while keeping the vascular supply and drainage at the base intact. The cremasteric artery enters at the base of the muscle and branches away from where the muscle pouch is opened so the supply is not disrupted (Gavins et al., 2004). Using blunt dissection the testis was separated from the cremaster muscle. The testis could then be pulled through the inguinal canal so that it did not obstruct the preparation. The muscle was then spread out onto a clear Sylgard disc (silicone 10:1 curing agent) using forceps ensuring only the edges are manipulated to avoid vascular damage. Micro pins were then used to secure the flattened muscle (see Fig. 2.1). The exposed muscle was kept moist using topical addition of physiological salt solution (PSS) via a pipette. The cardioplegic solution perfusion the circulation is then replaced with PSS with 0.1% albumin delivered by a gravity controlled reservoir.

2.1.5 Stage setup

The preparation was then moved to the microscope stage and the cremaster muscle was superfused with PSS gassed with 5% carbon dioxide at 37°C. The PSS pH of 7.40 was maintained by the buffering with the 5% carbon dioxide (95% air). The superfusate was added continually using a pump, with a flow rate of 2.5ml/min, and temperature controlled dripper over the tissue. Excess fluid was removed using suction. The PSS contained the Na^+ channel blocker lidocaine (20mg/l) to inhibit the cremaster muscle from contracting, which would affect recordings. The cremaster was continuously perfused with PSS with albumin. There was then a 30 minute equilibration period during which appropriate vessels for visualisation were located.

2.2 Experimental protocol

2.2.1 Vessel selection and viability

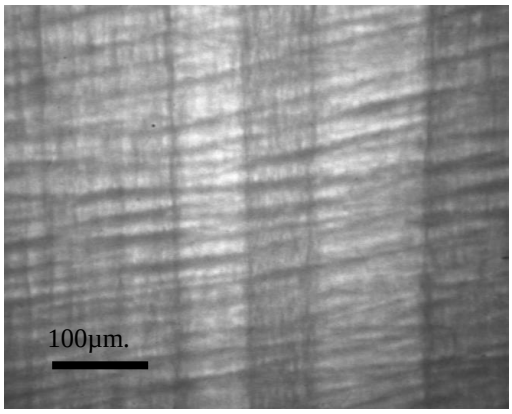

Figure 2.2. Arteriole and venule visualised under transilluminated light with x10 objective.

Following the set up of the stage, using transillumination a x10 magnification aqueous-immersion objective lens was used to visualise and select a vessel (see Fig. 2.2). As it is the EDHF response in arteries that are being investigated in this study, an appropriate artery was chosen. Arteries can be distinguished from veins by having a less torturous route, thicker walls but narrower lumens, and can be followed back to the main cremasteric artery. The vessels could be visualised through the microscope eye piece or through the computer screen connected to the camera. Using a stage micrometre, each objective lens was calibrated so that the diameters could be measured. Second order arterioles (diameter 50-100 microns) were chosen. To reduce the risk of damage, the vessels chosen were close to the centre of the tissue and away from the periphery which may have been handled during preparation. An EDHF mediated relaxation of >50% was considered to signal vessel viability (protocol described below). If the relaxation was below 50% the arteriole was not used and

viability was tested in a different vessel. The stability of the preparation over time was also measured using this viability procedure.

2.2.2 Pre-constriction

Following the 30 minutes equilibration time the arterioles were pre-constricted by superfusion with 30μM phenylephrine. This concentration of the selective α_1-adrenergic receptor gave a prolonged constriction. The α_1-adrenergic receptor is a Gq protein coupled receptor. Following its activation PLC is activated causing the release of the second messengers IP_3 and DAG leading to an increase in Ca^{2+} in the smooth muscle cell and constriction. 300μM N(G)-nitro-L-arginine methyl (L-NAME) and 3μM indomethacin were co-applied as NOS and COX inhibitors to block NO and PGI_2 mediated reactions respectively. This ensures only EDHF mediated relaxation were present. Unless otherwise stated the tissue was continually superfused with phenylephrine, L-NAME and indomethacin. There was a 20 minute equilibration time following application to ensure a stable baseline constriction and time for the inhibitors to work.

2.2.3 EDHF mediated relaxation

The stable acetylcholine analogue and muscarinic receptor agonist carbachol (10μM) was added to elicit EDHF mediated relaxation (see fig 2.2). The carbachol was added by superfusing for 2 minutes and gave a relaxation which was reversible following washout. 15 minutes was left between carbachol administrations to reduce tachyphylaxis and allow constriction back to baseline.

2.2.4 Drug application

During the experiments, phenylephrine, L-NAME and indomethacin were applied throughout. Unless otherwise stated drugs were applied through the superfusate. A bubble was used to show their arrival onto the tissue. To investigate the effect on EDHF responses, additional drugs were added to the tissue before carbachol was applied to allow for an appropriate incubation time (2-20 minutes). The drugs were then also co-applied with the carbachol. Following application there was then a 15 minute wash out period as long as the drugs effects are reversible. Following the addition of drugs the pH of the PSS superfusate was checked and maintained at pH 7.40.

2.3 Drugs and solutions.

The physiological salt solution (PSS) was made freshly each day and buffered to pH 7.40 using a pH meter. The PSS contained 124mM NaCl, 5mM KCl, 22mM $NaHCO_3$, 5mM glucose, 2mM $MgSO_4$, 0.125mM NaH_2PO_4, 2mM $CaCl_2$, gassed with 5% CO_2 - balance air to maintain the pH. The St. Thomas' cardioplegic solution contained 110mM NaCl, 7.9mM KCl 34mM $MgCl_2$, 1mM $CaCl_2$, 11mM HEPES, 10mM isoprenaline and the same weight of ascorbic acid as an antioxidant. The salts were purchased from VWR international and Sigma Aldrich.

Drugs were made up freshly each day or made from stock solutions stored at -20°C with final concentrations made up in PSS.

All drugs were purchased from Sigma Aldrich other than: TRPM2 antibody (Cambridge bioscience), $AACOCF_3$ (Merck Chemicals), Heparin (AAH Hospital Services), TRAM-34 (Tocris Bioscience), Lidocaine (Braun Medical), L-012 (Wako Chemicals), DCF and DHE (Invitrogen Life Technologies) and CP91 and CP85 which were kindly donated by Prof. Robert Hider (King's College London). Animals were supplied by Charles River.

2.4 Analysis

The EDHF response was investigated by measuring changes in diameter and endothelial cell Ca^{2+} following carbachol application. The effect of different drugs on the EDHF response could then be studied to investigate the role of ROS in the pathway.

2.4.1 Diameter measurements

The EDHF mediated relaxation to carbachol was measured using the Image Hopper software. Using the camera attached to the microscope, an image was taken once a second for 300 seconds which was then displayed on the video monitor producing a stack of images. This recording then allowed the change in diameter to be measured and calculate the % relaxation. A x40 aqueous-immersion objective lens was used for recordings. This allowed diameter measurements to be taken to an accuracy within 1 micron. The fluorescent indicator fluoresceinisothiocyanate (FITC)-albumin (5%) was added to the perfusate to allow for clear visualisation of the vessels by switching from

transillumination to 490-530nm illumination using the correct filter. Using image hopper to change the threshold of the captured fluorescent signal then allowed the image to be displayed with the vessels appearing white and the background black for easier differentiation (see fig 2.3).

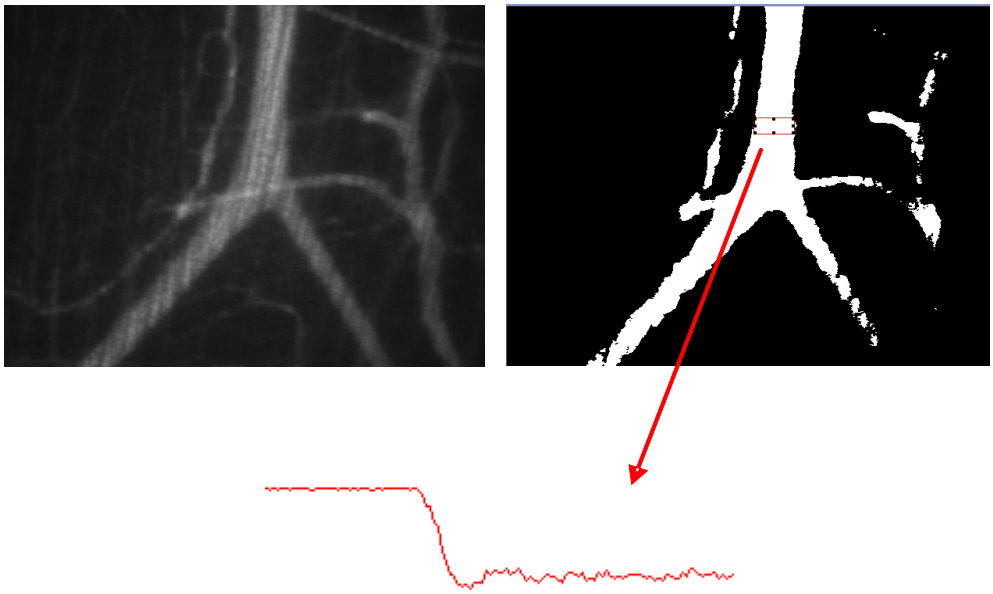

Figure 2.3. Dynamic change in diameter measured following carbachol application using Image Hopper. The red line represents the change in diameter of the vessel.

At the beginning of the preparation the baseline arteriole diameter was recorded before the administration of phenylephrine. Following phenylephrine application there was constriction of the arteriole. L-NAME and indomethacin were present to ensure only the EDHF response was being seen. The recordings started and following approximately 60 seconds carbachol was added leading to EDHF mediated relaxation. The maximum diameter following relaxation was then measured. To measure the diameter a box was drawn over the arteriole, which measures the diameter in pixels and shows the dynamic change in diameter as well as the diameter before and after carbachol was added (see Fig. 2.3). The diameter can be converted to microns by using a stage micrometer which showed 100 microns equals 310 pixels (for the x40 objective). The % relaxation is the dilatation to carbachol as a percentage of the full

dilatation of the arteriole. The EDHF response could be then characterised by measuring the % relaxation to carbachol with and without other drugs.

2.4.2 Calcium measurements

An increase in endothelial cell Ca^{2+} is a vital step in the EDHF response so this was measured simultaneously. The Ca^{2+} indicator Fura-PE3 AM, a dimer of Fura-2 AM, was used as it is cell permeant but then is protonated within the cell so does not leak out as easily as Fura-2 AM. At room temperature 10μM Fura-PE 3 AM was injected into the perfusate with 1% DMSO and 0.02% pluronic acid to aid dispersion in 1% albumin PSS. The mixture also included the dye FITC albumin so that perfusion could be monitored and diameter changes recorded. This mixture was allowed to perfuse for 1 hour to allow for good loading. Using UV light and the video camera clear 'tram lines' indicated good loading in the endothelial cells. The mixture was then washed through with PSS. The superfusate was then turned back to 37°C, after which there was a 15 minute equilibration period before starting experiments. A water immersion x40 objective was used for recordings. Recordings were made using Image Hopper with a filter wheel switching between 360nm and 380nm excitation each second. The 360/380 ratio provides a semi-quantitative estimate of endothelial cell $[Ca^{2+}]$, and was used rather than the more conventional 340/380 ratio due to fluorescence being too weak from 340nm excitation. The recordings were made simultaneously with the diameter recordings with 1 second intervals for 300 images producing a stack of images.

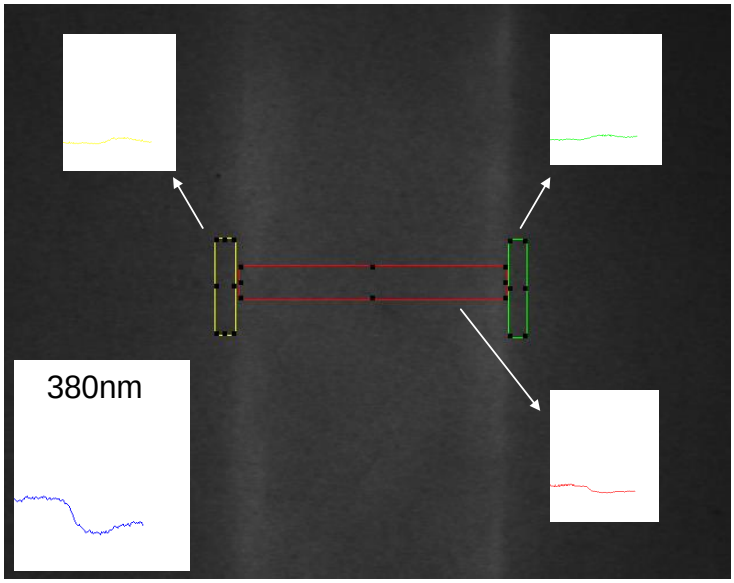

Figure 2.4. Image analysis of change in fluorescence.
Image analysis of emissions at 380nm – vessel recording (red) normalised to average of background (green and yellow) which is then subtracted. Produces change in fluorescence due to calcium increase in endothelial cell (blue) without background autofluorescence.

Changes in signal fluorescence were calculated using Image Hopper. Figure 2.4 shows the analysis of a stack of images. An average of the background (yellow and green) was taken. The background fluorescence is then subtracted from the image, and the signal from the vessel (red) was normalised, producing a graph (blue) showing the change in fluorescence at 380nm. This was repeated for the 360 recording and then a ratio was taken of the recordings. The % change in ratio is a semi-quantitative representation of the change in intracellular Ca^{2+}. This allowed changes in endothelial cell Ca^{2+} during the EDHF response to be measured and the effect of different drugs to be investigated.

2.4.3 Presentation of data and statistical analysis

Calcium measurement data is represented as the % increase in the Fura-PE3 360/380 ratio from the baseline, corresponding to the rise in intracellular Ca^{2+} in the

endothelium. The ratio increase is measured to the peak of the ratio rise. The diameter measurements are represented as the maximum % relaxation following carbachol administration. The response to carbachol alone is paired with the response to carbachol with another drug allow for comparison of the results. The paired results are always from the same experiment on the same arteriole. These paired results are represented in graphs showing how the EDHF mediated relaxation and endothelial cell Ca^{2+} increase to carbachol are affected by different drugs (GraphPad Prism).

The means $\pm$ the standard error have been calculated, with n= the number of experiments performed. A two-tailed paired t-test was used to analyse the results to measure if there was a statistically significant difference. This test is appropriate for paired data and where there is a normal distribution as there was for the % relaxation and % ratio increase. If the calculated p values were less than 0.05 the differences were considered significant as this means that there is more than a 95% probability the results were not due to chance.

Chapter 3: Characterising the response to carbachol in rat cremaster arterioles.

3.1 Introduction

3.1.1 Carbachol response

In this chapter, I describe experiments that were performed to characterise the relaxation response to the muscarinic receptor agonist carbachol in rat cremaster arterioles. The EDHF mediated component of this relaxation was further investigated so that the underlying mechanism could be understood. Control experiments were carried out to ensure the results were reproducible and reliable. A pharmacological approach was then used with different channel and enzyme inhibitors so their involvement in the EDHF mediated relaxation pathway could be demonstrated.

Carbachol was used as a muscarinic receptor agonist to elicit relaxation in the arterioles. Carbachol was used rather than acetylcholine because it is a more stable analogue as it is not easily metabolised by cholinesterases (Hamilton et al., 2001). Muscarinic receptor agonists have been demonstrated to cause an EDHF relaxation in a range of tissues and are frequently used to study the mechanisms involved (Dong et al., 2000; Hatoum et al., 2005). In small resistance vessels like the arterioles used in this study, it has been previously demonstrated that the relaxation to acetylcholine and its analogues is mainly due to the EDHF pathway (Bryan et al., 2005; Jin et al., 2008). In larger arteries NO seems to be the dominant mechanism, but in smaller vessels the EDHF pathway is the main relaxation mediator (Garland et al., 1995). This was shown by the relaxation being maintained even in the presence of L-NAME and indomethacin to block the NO and PGI_2 relaxation pathways (Shimowakaet al., 1996).

3.1.2 Characterising the EDHF response

The EDHF response to carbachol was characterised in the rat cremaster arterioles. The response is characterised by the opening of the IK_{Ca} and SK_{Ca} channels on the endothelium (Andersson et al., 2000). Sensitivity to the blockers of the IK_{Ca} and SK_{Ca} channels allows the EDHF response to be targeted. Apamin can be used to selectively block the SK_{Ca} channels, while TRAM-34 is a synthetic inhibitor of the IK_{Ca} channel. This was used in preference over charybdotoxin which is used in other studies as TRAM-34 is selective for the IK_{Ca} channel whereas charybdotoxin also blocks the BK_{Ca} channels as well (Gluais et al., 2005). The EDHF response can also be mimicked by using a selective and potent IK_{Ca} and SK_{Ca} channel opener, NS309 (Tharp & Bowles, 2009). This causes an endothelium dependent hyperpolarisation (Leuranguer et al., 2008), allowing the specific characterisation of this part of the relaxation pathway as it directly targets a downstream process. During NO and PGI_2 dependent relaxation, protein kinase G is activated by cGMP production. PKG causes relaxation of the smooth muscle by lowering intracellular Ca^{2+}. To investigate whether PKG is also involved in the EDHF response the PKG inhibitor Rp-8-bromo-cGMP was used (Yao et al., 2000).

3.1.3 Gap junctions and the EDHF response

Different pathways have been shown to be involved in the EDHF mediated relaxation, including a role for myoendothelial gap junctions (MEGJs). MEGJs may be acting as part of an EDHF process rather than there being a single endothelial derived factor that acts directly on the smooth muscle cell to cause relaxation (Griffith, 2004). The

role of MEGJs can be investigated using inhibitors that can block the spread of the electrical current through the channels. Using gap junction inhibitors, the EDHF response has been abolished in different arteries suggesting it has a key role in the relaxation pathway (Sandow et al 2002; Mather et al., 2005). 18α-GA has been shown to block gap junction communication between cells so can be used to study the importance of the channels (Jin 2008). Its affects can be irreversible but it can be used at concentrations that do not directly affect smooth muscle tone (Griffith, 2004).

3.1.4 Endothelial cell Ca^{2+}

An increase in endothelial cell [Ca^{2+}] is a key process in the EDHF mediated relaxation. This increase causes the activation of the IK_{Ca} and SK_{Ca} channels leading to the hyperpolarisation of the endothelial cell due to the K^+ efflux. Intracellular [Ca^{2+}] can increase by influx of extracellular Ca^{2+} through channels on the cell membrane, or by release of Ca^{2+} from internal stores. This is regulated by the IP_3 receptor on the endoplasmic reticulum. To investigate the importance of these mechanisms different Ca^{2+} channel blockers were used. SK&F 96365 is a non-selective inhibitor of receptor operated Ca^{2+} channels and some of the TRP family of cation channels so can be used to investigate extracellular Ca^{2+} entry (Groschner et al., 1994). This study is looking at the role of redox signalling and TRPM2 has been shown to be redox sensitive. The TRPM2 antibody has been used to selectively block the TRPM2 channel so it was used to study its role (Hecquet et al., 2008). Ca^{2+} release from internal stores was studied by using a Ca^{2+} free PSS containing EGTA, under which condition Ca^{2+} influx is eliminated, whereas release is preserved. To further investigate Ca^{2+} release, I used the the phospholipase C inhibitor U73122, as

well as its inactive analogue U73343 (Volk et al., 1997). PLC causes the release of the signalling molecule IP_3 from the cell membrane so its inhibition blocks the store release of Ca^{2+}.

3.2 Methods

The basic response to carbachol in the rat cremaster arterioles was characterised using intravital microscopy allowing for changes in diameter and endothelial cell Ca^{2+} to be measured. The tissue preparation and intravital microscopy setup is described in the previous General Methods chapter.

3.2.1 Protocol

The standard protocol was that the arterioles were pre-constricted with 30µM phenylephrine in the presence of 300µM L-NAME and 3µM indomethacin. To characterise the EDHF response, 10µM carbachol was added for 2 minutes and changes to the diameter and endothelial cell Ca^{2+} were measured. This experiment was then repeated in the presence of different channel and enzyme inhibitors to understand the mechanisms involved in the EDHF response. The inhibitors were added to the preparation prior to application of carbachol. The pre-treatment with the inhibitors was for 2 minutes other than 20 minutes for L-NAME, 5 minutes for apamin, TRAM-34, U73122 and U73343, 15 minutes for 18α-GA and 1 hour perfusion for the TRPM2 antibody. Between experiments there was a 15 minute wash out period other than with SKF-96365, 18α-GA and the TRPM2 antibody which were

found not to be fully reversible. Experiments with inhibitors that were not fully reversible were done at the end of the run of experiments.

3.2.2 Ca^{2+} free preparation

Some experiments were performed in a Ca^{2+} free preparation to ensure there was no influx of extracellular Ca^{2+} into the endothelial cells during the EDHF response. This allowed the Ca^{2+} release from internal stores to be studied. For Ca^{2+} free recordings a Ca^{2+} free PSS with 0.2mM ethylene glycol tetraacetic acid (EGTA), a Ca^{2+} chelator, replaced the normal PSS in the perfusate and superfusate. The preparation was left for 15 minutes to allow it to become Ca^{2+} free. All drugs that were added were made up in solutions of the Ca^{2+} free PSS. Following a recording normal PSS with Ca^{2+} was applied for 15 minutes to allow the internal Ca^{2+} stores to be replenished before switching back to the Ca^{2+} free solutions to carry out another experiment. The pre-constriction with phenylephrine was abolished in the Ca^{2+} free preparation so only changes in endothelial cell Ca^{2+} were measured.

3.2.3 Control experiments

Time control experiments were performed to monitor the stability of the responses to carbachol over time and to ensure they were reproducible and reliable (see 3.3 results section). Time controls were also performed to ensure the stability of the phenylephrine induced constriction to make sure that there was a stable baseline and no spontaneous relaxation. The different inhibitors and drugs were applied to the

arterioles before the addition of carbachol so that any direct effects of the drugs on the diameter or endothelial cell [Ca^{2+}] could be observed.

3.2.4 Analysis

The responses to carbachol (% relaxation and % ratio increase) are paired with the response with the inhibitors present in the same arteriole to allow for direct comparison. These paired results are displayed in the graphs and were analysed with a two-tailed paired t-test. The difference between the compared results was considered significant if the p value was less than 0.05, represented by a * on the graph (** $p<0.01$, *** $p<0.001$).

3.3 Results

3.3.1 Controls

Control experiments were carried out to ensure the stability of the response to 30µM phenylephrine. Figure 3.1 shows a typical trace of the constriction to phenylephrine in the presence of 300µM L-NAME and 3µM indomethacin. This produced a stable constriction (32.9% $\pm$ 1.9, n=55) that did not run down over time. Controls were also performed following the addition of 10µM carbachol to ensure a suitable relaxation was elicited that was prolonged enough to take an accurate reading of the maximum diameter. Figure 3.2 shows a typical trace with carbachol producing an immediate relaxation that gradually diminished down over time. A carbachol dose response curve showed that 10µM carbachol caused almost maximal relaxation with no further increase at 30µM or 100µM carbachol (see Fig. 3.3). The % relaxation to 3µM carbachol was only around 25% so 10µM was chosen as the lowest concentration that gave a consistent significant relaxation.

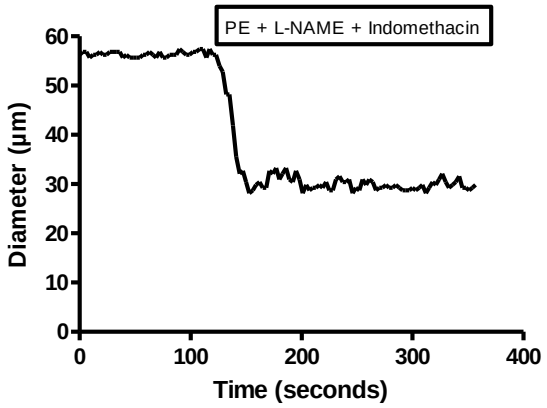

Figure 3.1. Typical trace of the constriction to phenylephrine.

The addition of 30µM phenylephrine, 300µM L-NAME and 3µM indomethacin caused a sustained constriction. There was a 32.9% ± 1.9 (n=55) constriction of the maximum diameter.

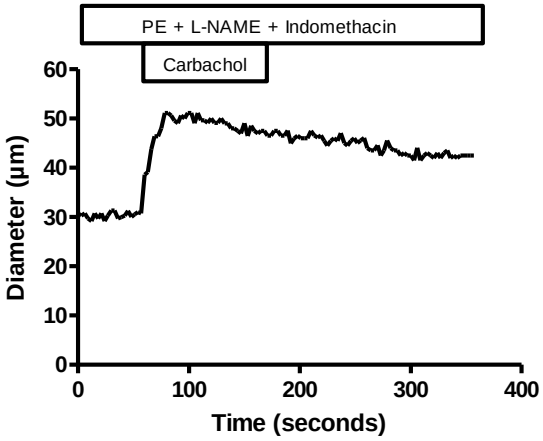

Figure 3.2. Typical trace of the relaxation to carbachol.

The addition of 10µM carbachol for 2 minutes, in the presence of PE, L-NAME and indomethacin, caused EDHF-mediated relaxation of 72.6% ± 2.4 (n=55).

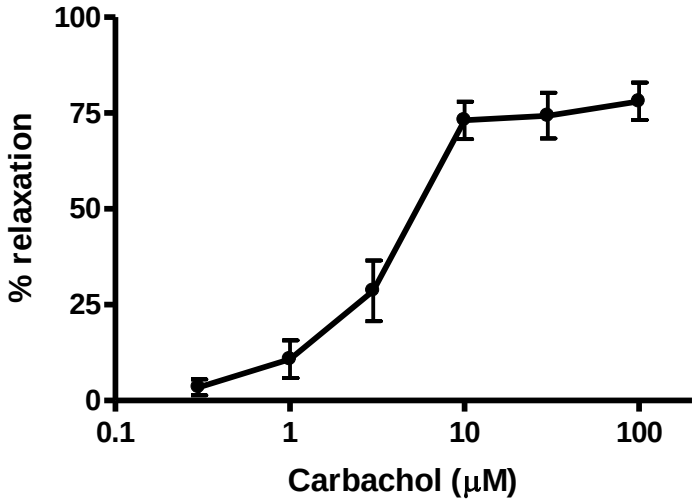

Figure 3.3. Concentration dependent relaxation to carbachol of PE-preconstricted rat cremaster arterioles.

Graph shows the % relaxation to carbachol (0.1-100μM) in the presence of PE, L-NAME and indomethacin. Results are mean ± standard error, n=5.

3.3.2 Time controls

Time controls were performed by recording the % relaxation and % ratio change (i.e. change in the ratio of fluorescence emission at 340 and 380nm excitation) following two carbachol applications separated by 15 minutes. These experiments (see Fig. 3.4) showed that there was not a significant difference in the EDHF mediated relaxation in response to carbachol (75.3% $\pm$ 3.5 on first application compared to 72.5% $\pm$ 3.2 on second application, n=21, n.s). Moreover, the % change in ratio in response to carbachol also did not diminish over time (see Fig. 3.5). In the Ca^{2+} free preparation there was a transient rise in endothelial cell [Ca^{2+}] which, following incubation in normal - [Ca^{2+}] PSS for 15 min to allow store refilling, was reproducible when carbachol was again applied under Ca^{2+} free conditions, with no significant difference being observed between the two applications of carbachol (see Fig. 3.6) showing Ca^{2+} responses are stable. These results show the effect of carbachol was stable when the standard protocol was used, indicating that this approach was suitable for assessing the effects of various drugs on the response.

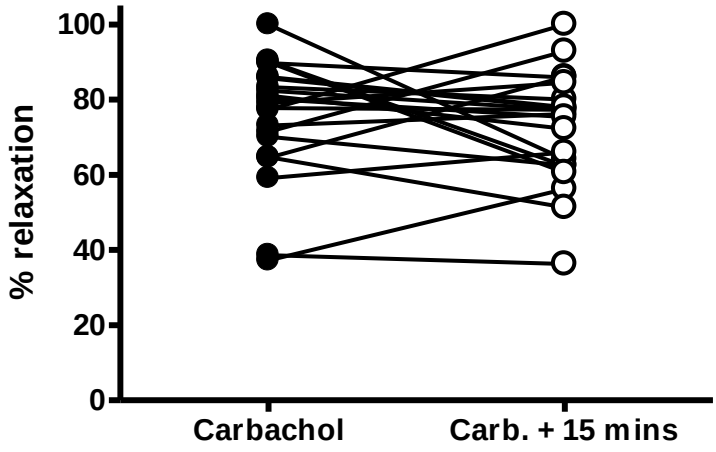

Figure 3.4. Time controls for the relaxation to repeated carbachol applications.

The relaxation to 10µM carbachol addition (75.3 % ± 3.5) was maintained with repeated carbachol application (72.5 % ± 3.2). Results show mean ± SE, n=21 , (n.s).

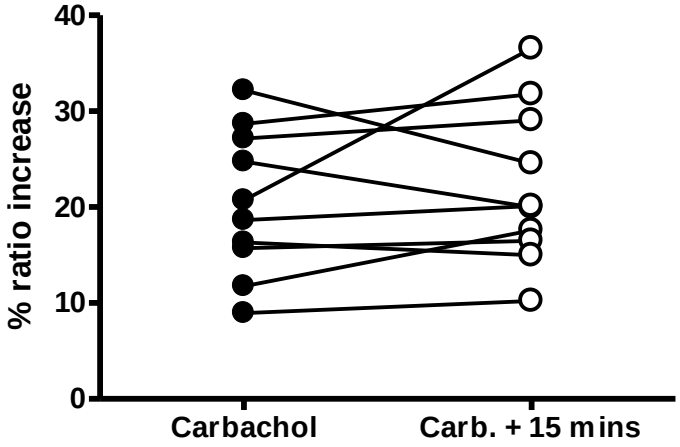

Figure 3.5. Time controls for the 360/380nm ratio increase to repeated carbachol applications.

The ratio increase to 10µM carbachol addition (20.5 % ± 2.5) was maintained with repeated applications (22.1 % ± 2.6). Results show mean ± SE, n= 10, (n.s)

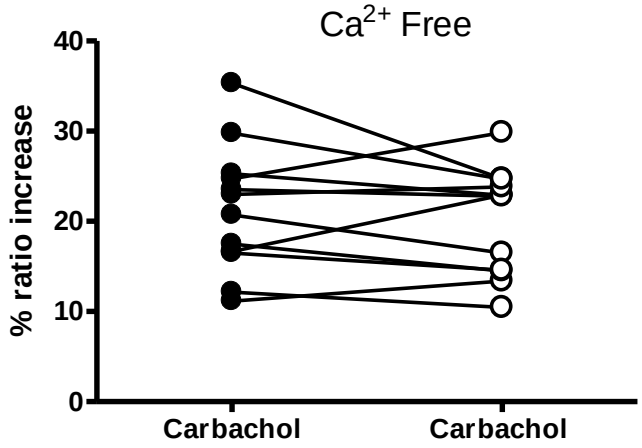

Figure 3.6. Time controls for the 360/380nm ratio increase to carbachol in Ca^{2+} free preparation.

In the Ca^{2+} free preparation the increase in the ratio is maintained with repeated carbachol applications (from 21.4 % $\pm$ 2.0 to 20.1 % $\pm$ 1.7. Results show mean $\pm$ SE, n= 12, (n.s).

3.3.3 Relaxation pathways in the rat cremaster arteriole

10μM carbachol was added with and without different inhibitors in order to assess the contribution of EDHF to endothelium-dependent vasodilation (see Fig. 3.7). The addition of carbachol alone (without L-NAME and indomethacin present) caused almost complete relaxation. Blocking the NO and PGI_2 pathways with L-NAME and indomethacin only slightly (but significantly) inhibited relaxation. When the EDHF pathway was blocked with SK_{Ca} and IK_{Ca} channel blockers apamin and TRAM-34, there was >80% inhibition of the L-NAME and indomethacin independent relaxation, showing the importance of the EDHF pathway in these vessels. When apamin and TRAM-34 were applied alone, without L-NAME and indomethacin the relaxation to carbachol was reduced from 95.7 % $\pm$ 1.2 to only 22.4 % $\pm$ 1.4 (not shown). This shows that the EDHF response is not just in response to NO and PGI_2 inhibition and is always present.

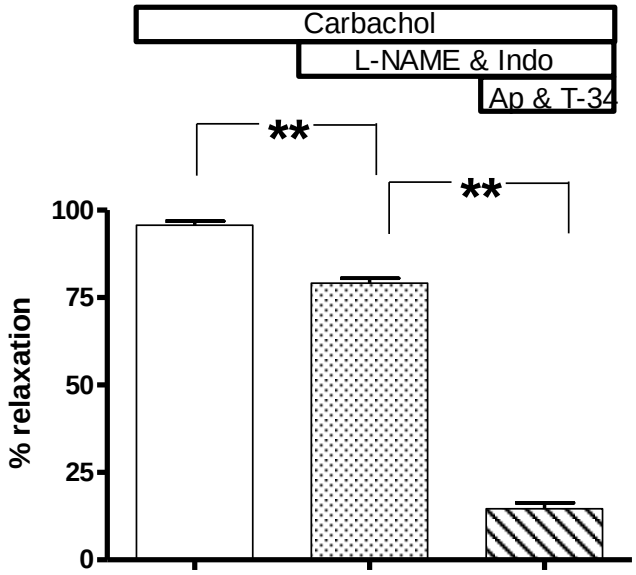

Figure 3.7. Relative contribution of relaxation pathways in rat cremaster arterioles.

The relaxation of PE-preconstricted cremaster arterioles to carbachol (95.7 % ± 1.2) was attenuated by the NOS and COX inhibitors L-NAME + indomethacin (79.1 % ± 1.4). The addition of the SK_{Ca} and IK_{Ca} antagonists apamin (500nM) and TRAM-34 (10μM) which block the EDHF response significantly inhibited the relaxation (14.7 % ± 1.8). Results show mean ± SE, n=5 , ** $p < 0.01$.

3.3.4 The role of Ca^{2+} channels.

SK&F 96365 inhibits Ca^{2+} entry through receptor mediated Ca^{2+} channels and some non-specific cation channels such as TRP channels. EDHF-mediated relaxation to carbachol was inhibited by 10μM SK&F 96365 (see Fig. 3.8). SK&F 96365 also caused a significant inhibition of the $[Ca^{2+}]_i$ rise in response to carbachol during EDHF induced relaxation (see Fig. 3.9), with an almost 50% reduction in the ratio increase. As would be expected, in the Ca^{2+} free preparation the SK&F 96365 did not affect the % ratio increase as the $[Ca^{2+}]_i$ rise under these conditions is entirely due to release from internal stores (results not shown). TRPM2 is a cation channel present on endothelial cells which is thought to be ROS sensitive. Pre-treatment with a TRPM2 antibody that blocks the channel specifically however had no effect on the EDHF response to carbachol with no inhibition of the relaxation or endothelial cell Ca^{2+} rise (see Fig. 3.10 & 3.11).

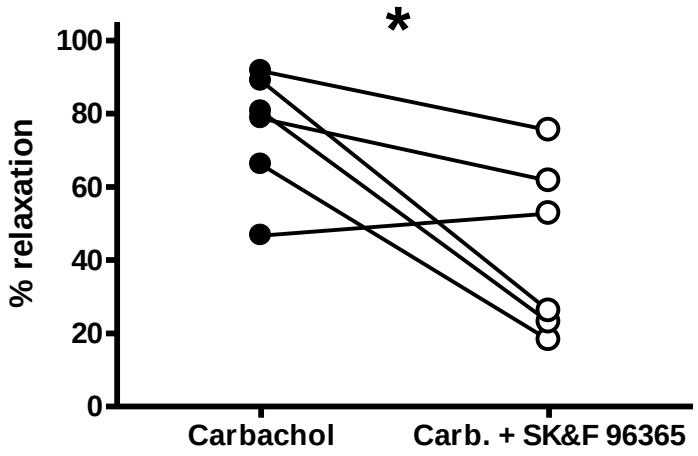

Figure 3.8. The effect of SK&F 96365 on relaxation to carbachol.

The relaxation of PE-preconstricted cremaster arterioles to carbachol (75.4 % ± 6.8) was inhibited by the receptor operated Ca^{2+} channel blocker SK&F 96365 (10μM) (42.9 % ± 9.7). Results show mean ± SE, n=6 , * $p < 0.05$.

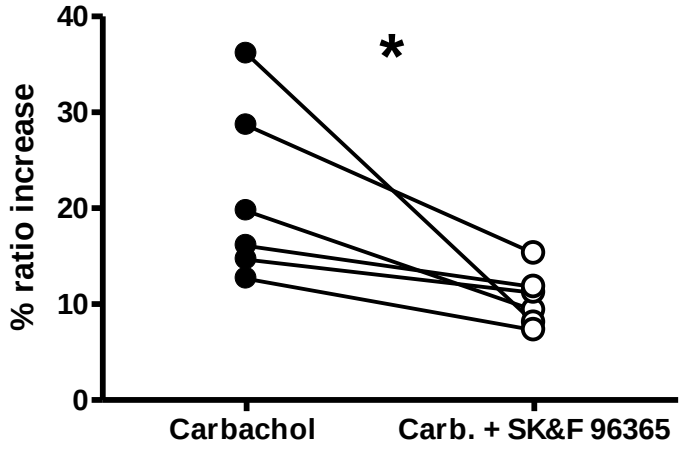

Figure 3.9. The effect of SK&F 96365 on the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (21.3 % ± 3.8) was inhibited by the receptor operated Ca^{2+} channel blocker SK&F 96365 (10μM) (10.5 % ± 1.2). Results show mean ± SE, n=6 , * $p < 0.05$.

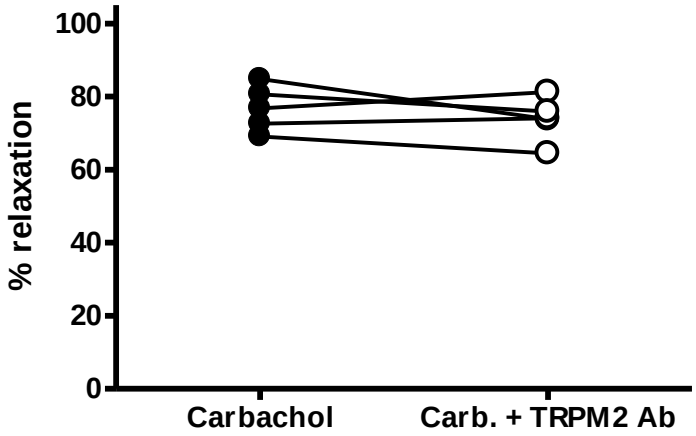

Figure 3.10. The role of TRPM2 channels in the relaxation to carbachol.

The relaxation of PE-preconstricted cremaster arterioles to carbachol (76.8 % ± 2.8) was not affected by the TRPM2 antibody (5µg/ml) which blocks the TRPM2 channel (73.9% ± 2.7). Results show mean ± SE, n=5, (n.s).

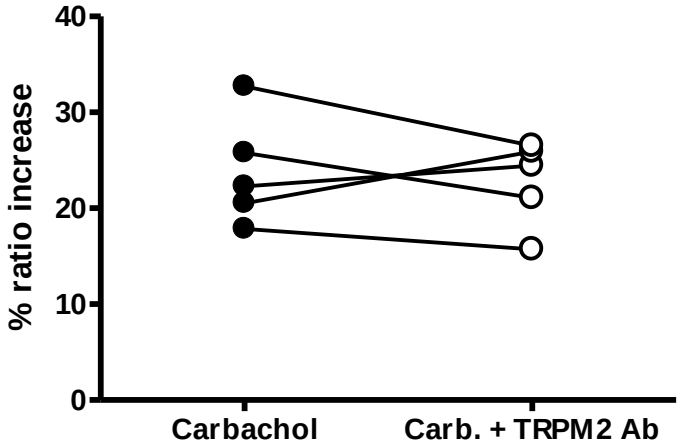

Figure 3.11. The role of TRPM2 channels in the 360/380nm ratio increase to carbachol.

The ratio increase to carbachol (23.8 % ± 2.6) was not significantly affected by the TRPM2 antibody (5µg/ml) which blocks the TRPM2 channel (22.7 % ± 2.0). Results show mean ± SE, n=5, (n.s).

3.3.5 The role of K^+ channels

As previously described, blocking the IK_{Ca} and SK_{Ca} channels caused a significant inhibition of the EDHF response to carbachol, almost totally abolishing the relaxation and also reducing the endothelial cell [Ca^{2+}] rise, albeit to a lesser degree (see Fig. 3.12 & 3.13). The importance of the channels was confirmed by using NS309, a selective IK_{Ca} and SK_{Ca} channel opener. This directly caused a relaxation which though smaller than the one elicited by carbachol was still significant (65.2% $\pm$ 1.8, n=6). NS309 also caused a small increase in endothelial cell $[Ca^{2+}]_i$ (Fig. 3.18 and 3.19). The BK_{Ca} channel blocker iberiotoxin however had no affect on the EDHF response to carbachol with no inhibition of relaxation of the endothelial cell Ca^{2+} increase (see Fig. 3.14 & 3.15).

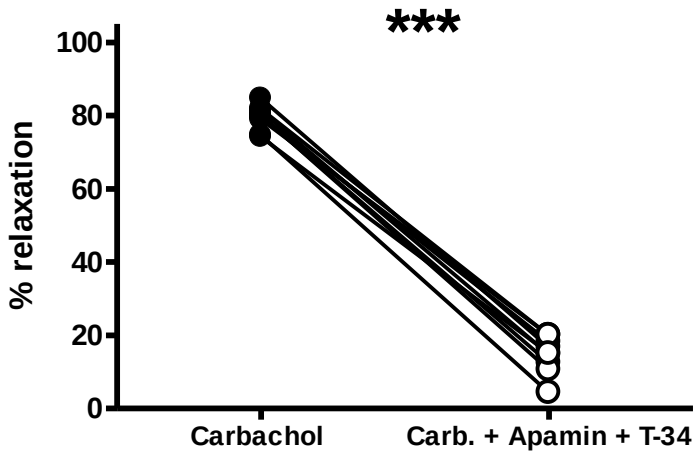

Figure 3.12. The effect of apamin and TRAM-34 inhibition on the relaxation to carbachol.

The % relaxation to carbachol (79.4 % ± 1.1) was almost abolished by the SK_{Ca} and IK_{Ca} blockers apamin (500nM) and TRAM-34 (10μM) (14.7 % ± 1.8). Results show mean ± SE, n= 9, *** $p < 0.001$.

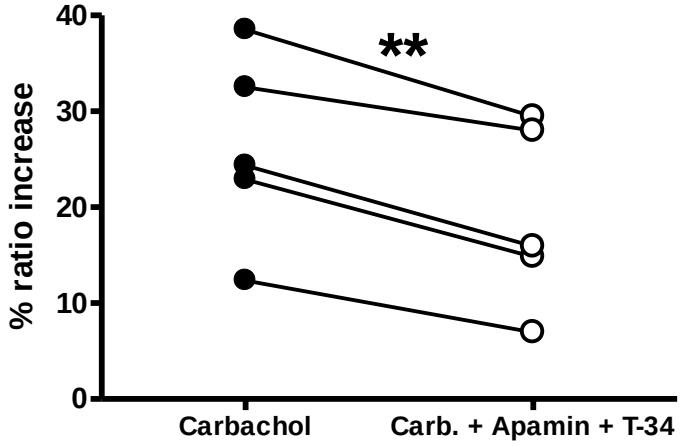

Figure 3.13. The effect of apamin and TRAM-34 on the ratio increase to carbachol.

The % 360/380nm ratio increase to carbachol (26.1 % ± 4.5) was inhibited by the SK_{Ca} and IK_{Ca} blockers apamin (500nM) and TRAM-34 (10μM) (19.0 % ± 4.3). Results show mean ± SE, n=5 , ** $p < 0.01$.

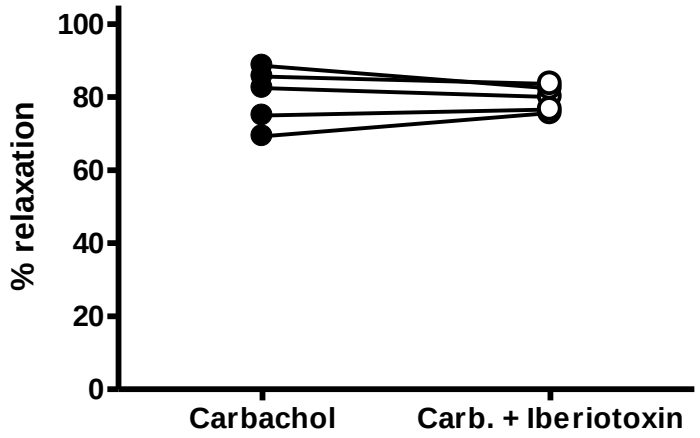

Figure 3.14. The role of BK_{Ca} channels in the relaxation to carbachol.

The relaxation to carbachol (80.2 % $\pm$ 3.6) was not significantly affected by the BK_{Ca} channel blocker iberiotoxin (100nM) (79.7 % $\pm$ 1.6). Results show mean $\pm$ SE, n=5, (n.s).

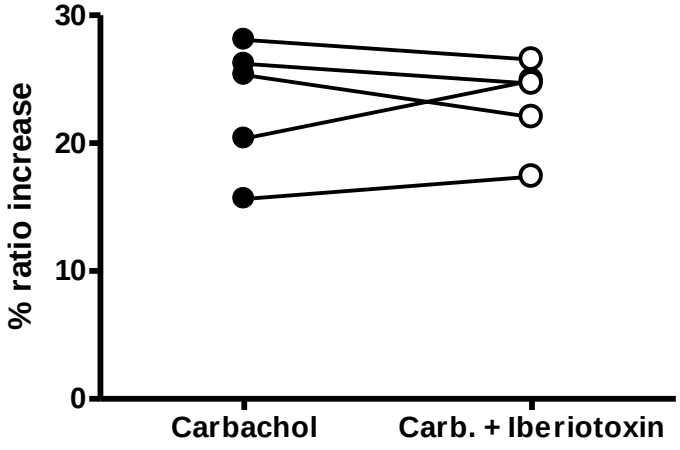

Figure 3.15. The role of BK_{Ca} channels on the ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (23.1 % $\pm$ 2.3) was not significantly changed by the BK_{Ca} channel blocker iberiotoxin (100nM) (23.1 % $\pm$ 1.6). Results show mean $\pm$ SE, n=5, (n.s).

3.3.6 The role of gap junctions

Gap junctions have been suggested to have an important role in the EDHF pathway. To investigate this, the gap junction inhibitor 18α-GA was applied to see the effect on the response to carbachol. The gap junction blocker almost completely abolished the relaxation to carbachol while having no effect on the endothelial cell Ca^{2+} (see Fig. 3.16 & 3.17). NS309 mimics the EDHF response by opening the IK_{Ca} and SK_{Ca} channels which will lead to hyperpolarisation of the endothelial cell. Similar to the effect on the response to carbachol, 18α-GA almost completely inhibited the relaxation to NS309 while having no effect on the endothelial cell $[Ca^{2+}]$ rise (see Fig. 3.18 & 3.19). This suggests that the role of gap junctions is downstream from the opening of the IK_{Ca} and SK_{Ca} channels and is a vital step in the pathway that leads to relaxation of the smooth muscle cell.

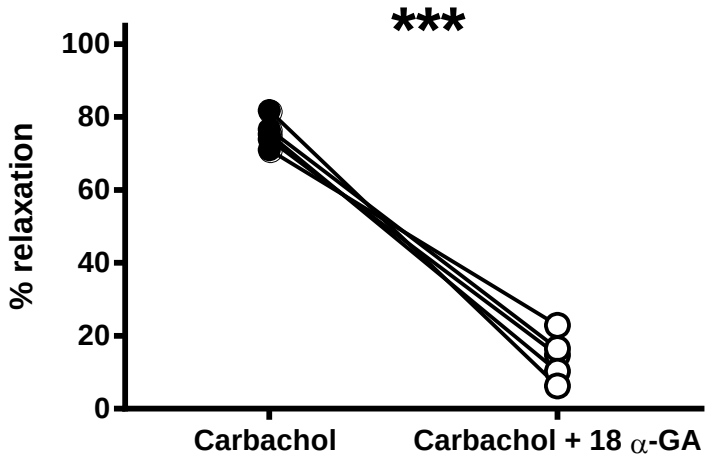

Figure 3.16. The role of gap junctions on the relaxation to carbachol.

The relaxation to carbachol (75.7 % ± 1.8) was significantly inhibited by the gap junction blocker 18α-GA (10µM) (14.1 % ± 2.8). Results show mean ± SE, n=5, *** $p < 0.001$.

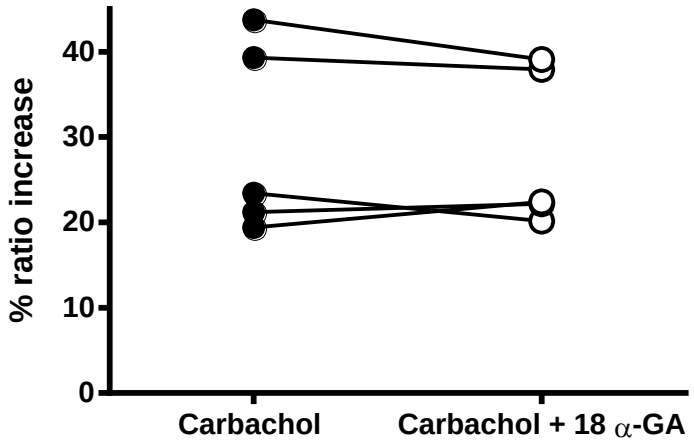

Figure 3.17. The role of gap junctions on the ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (29.4 % ± 5.0) was not significantly affected by the gap junction blocker 18α-GA (10µM) (28.4 % ± 4.2). Results show mean ± SE, n=5, (n.s).

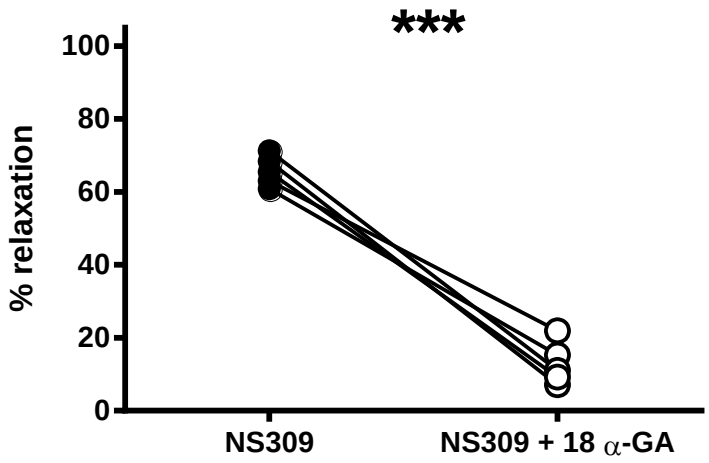

Figure 3.18. The role of gap junctions in the NS309 induced relaxation.

The relaxation to NS309 (10µM) (65.8 % ± 1.9) was almost abolished by the gap junction inhibitor 18α-GA (10µM) (12.9 % ± 2.6). Results show mean ± SE, n=5, *** $p < 0.001$.

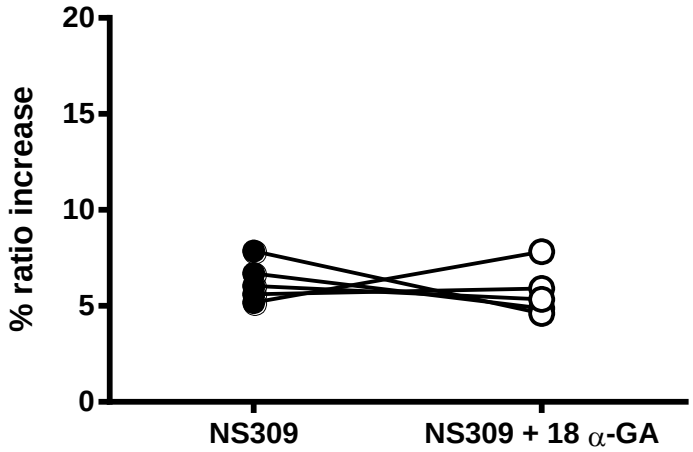

Figure 3.19. The role of gap junctions in the ratio increase to NS309.

The 360/380nm ratio increase to NS309 (10µM) (6.3 % ± 0.5) was not significantly affected by the gap junction blocker 18α-GA (10µM) (5.7 % ± 0.6). Results show mean ± SE, n=5, (n.s).

3.3.7 The role of PKG in the EDHF response

Protein kinase G (PKG) is an important enzyme in the relaxation pathway of NO and also potentially PGI_2, as cAMP can stimulate PKG (Caravajal et al., 2000). To investigate its role in the EDHF pathway the selective PKG inhibitor Rp-8-Bromo-cGMP was added to the preparation. The PKG inhibitor had no effect on the EDHF mediated relaxation to carbachol with the relaxation and endothelial cell [Ca^{2+}] rise maintained (see Fig. 3.20 & 3.21). This is consistent with the concept that EDHF works by a pathway different from those activated by NO and PGI_2 in causing VSM relaxation.

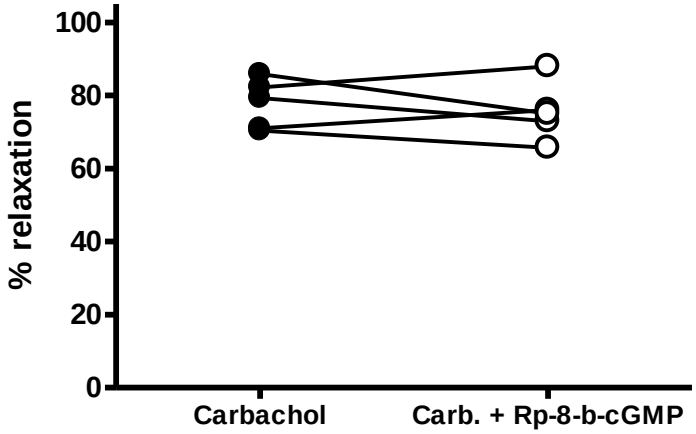

Figure 3.20. The role of PKG in the carbachol mediated relaxation.

The relaxation to carbachol (77.8 % ± 3.1) was not significantly affected by the PKG blocker Rp-8-bromo-cGMP (100µM) (75.5 % ± 3.6). Results show mean ± SE, n=5, (n.s).

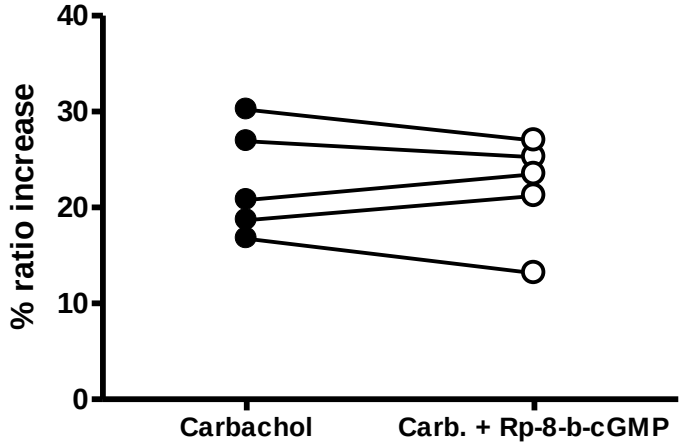

Figure 3.21. The role of PKG in the ratio increase following carbachol application.

The 360/380nm ratio increase to carbachol (22.7 % ± 2.5) was not affected by the PKG blocker Rp-8-bromo-cGMP (100µM) (22.0 % ± 2.4). Results show mean ± SE, n=5, (n.s).

3.3.8 The role of PLC in the EDHF response

Phospholipase C (PLC) is an important enzyme involved in signal transduction which can lead to the release of Ca^{2+} from internal stores that then has other downstream effects, and can be selectively inhibited by the drug U73122. Since this might have non-specific effects, an inactive analogue U73443 was applied as a negative control in other experiments. Whereas U73443 had no effect on either EDHF mediated relaxation or the associated increase in endothelial cell [Ca^{2+}] (Fig. 3.22 & 3.23), both responses were almost completely abolished in the presence of U73443 (Fig. 3.24 & 3.25). This suggests that the activation of PLC is a vital step in the EDHF pathway and that the rise in endothelial cell Ca^{2+} requires store release.

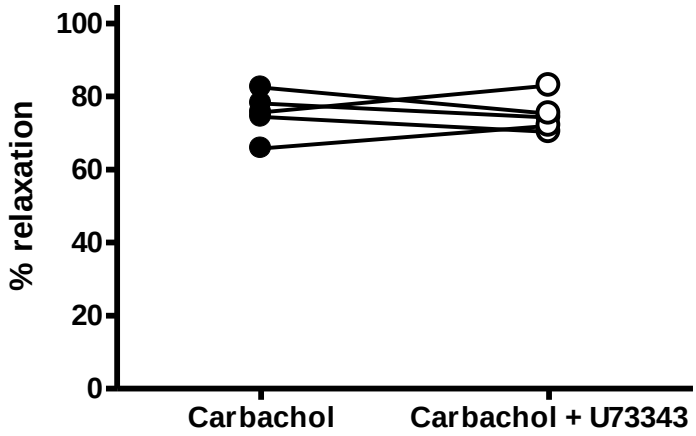

Figure 3.22. The affect of U73343 on the relaxation to carbachol.

The relaxation to carbachol (75.3 % ± 2.7) was not affected by U73343 (3µM), the inactive analogue of the PLC inhibitor U73122 which acts as a negative control (75.0 % ± 2.2). Results show mean ± SE, n=5, (n.s).

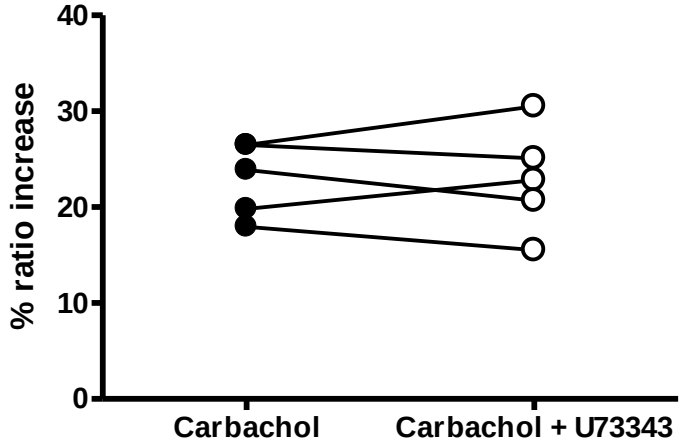

Figure 3.23. The affect of U73343 on the ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (22.9 % ± 1.7) was unaffected by U73343 (3µM), the inactive analogue of the PLC inhibitor U73122 (22.9 % ± 2.5). Results show mean ± SE, n=5, (n.s).

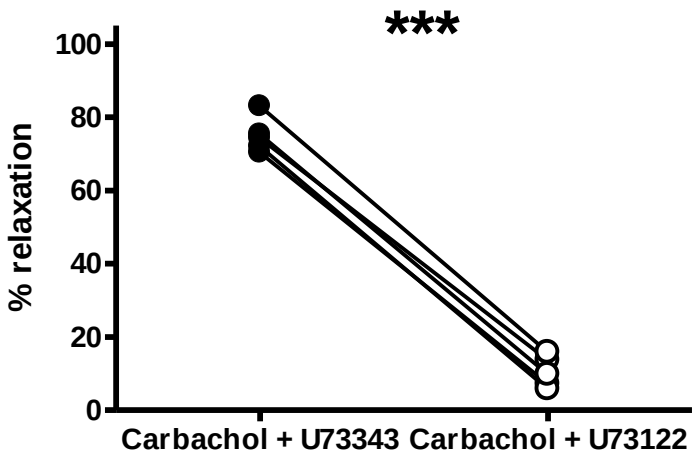

Figure 3.24. Comparison of the effects of U73343 and U73122 on the relaxation to carbachol.

The relaxation to carbachol (with U73343 as inactive analogue) (75.0 % $\pm$ 2.2) was almost abolished by PLC inhibition by U73122 (3μM) (10.4 % $\pm$ 1.9). Results show mean $\pm$ SE, n=5, *** $p < 0.001$.

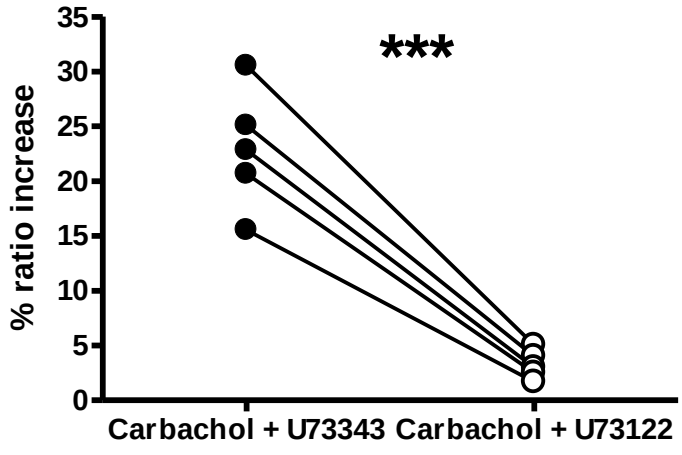

Figure 3.25. Comparison of the effects of U73343 and U73122 on 360/380 ratio.

The 360/380nm ratio increase to carbachol (with U73343 as inactive analogue) (22.9 % $\pm$ 2.5) was significantly inhibited by the PLC blocker U73122 (3μM) (3.2 % $\pm$ 0.6). Results show mean $\pm$ SE, n=5, *** $p < 0.001$.

3.4 Discussion

3.4.1 Response to carbachol in rat cremaster arterioles

The results presented in this chapter demonstrate that EDHF was the dominant mechanism for endothelium dependent vasodilatation, since L-NAME and indomethacin, which block NO and PGI_2 production respectively, only slightly reduced the relaxation to carbachol. This implied that the EDHF mediated relaxation amounted to ~80% of the overall response. The addition of apamin and TRAM-34, which block the SK_{Ca} and IK_{Ca} channels that are involved in the EDHF response, almost abolished the relaxation to carbachol, confirming that the L-NAME- and indomethacin-resistant relaxation was EDHF mediated. This supports the idea that in small vessels such as arterioles, EDHF is the prime endothelium dependent relaxation mechanism (Gluais et al., 2005), and is consistent with a previous study in rat cremaster arterioles where 70% of the relaxation to acetylcholine was found to be resistant to L-NNA, another NOS inhibitor (Bakker et al., 1997). The EDHF response worked via a different pathway to NO and PGI_2 as the PKG inhibitor Rp-8-bromo-cGMP had no effect on the response, whereas PKG is a major mediator of relaxation for these pathways by reducing Ca^{2+} in the VSM (Hare et al., 2003).

The EDHF vasodilatation to carbachol seemed to be mainly mediated through opening of the IK_{Ca} and SK_{Ca} channels, with the inhibition of the BK_{Ca} channel having no effect on the vasodilatation or the rise in endothelial cell $[Ca^{2+}]$. This supports the view that the BK_{Ca} channel is not involved in the EDHF response (Coleman et al., 2004). However, others have shown that activation of the BK_{Ca}

channel is important in tissues such as in the small mesenteric arteries in mice (Huang et al., 2001). This suggests that there could be species and tissue differences between the mechanisms involved in the EDHF response. There could also be differences depending on the agonist used with one study noting that the EDHF response to bradykinin involved activation of the BK_{Ca} channel, while the response to acetylcholine was not sensitive to BK_{Ca} blockade (Pannirselvam et al., 2006).

The relaxation and increase in endothelial cell [Ca^{2+}] in response to carbachol was shown to be sustained and repeatable, as demonstrated by the time controls in which there was no decrement in these responses over time. In addition to being consistent with the pathway having an important physiological role, the stability of the responses upon multiple applications of carbachol also meant that each arteriole could be used as its own control, allowing paired results to be analysed from the experiments. A dose response to carbachol showed that 10μM caused an almost maximal relaxation of ~80% so this concentration was used throughout for the experiments.

The rise in endothelial cell [Ca^{2+}] is known to be key to the EDHF response. It was found that using SK&F 96365 which blocked receptor operated Ca^{2+} channels and some members of the TRP family of cation channels significantly blocked the rise in endothelial [Ca^{2+}], and correspondingly blocked the relaxation. This supports other studies which have shown the importance of extracellular Ca^{2+} influx in the EDHF response (Doan et al., 1994). However, the TRPM2 antibody was used to specifically block the TRPM2 channel and this was found to have no effect on the EDHF response. Previous studies have described the role of TRPM2 in Ca^{2+} entry but none have looked specifically as its role in the EDHF response (Hecquet et al., 2008).

A Ca^{2+} free preparation was used to look at release of Ca^{2+} from intracellular stores in the endothelial cells. This produced a transient rise in [Ca^{2+}] in response to carbachol, consistent with store release without any extracellular influx. The stores could be replenished by the addition of the normal buffer with Ca^{2+} and then returned to Ca^{2+} free conditions to re-examine the effect of carbachol, and using this approach I found that the amplitude of this response was maintained. This was important, because it allowed the effects of drugs on Ca^{2+} release from stores to be investigated, as described in later chapters. The role of Ca^{2+} release was also investigated (in the presence of extracellular Ca^{2+}) using the PLC inhibitor U73122. This blocks PLC from releasing the second messenger IP_3 from the cell membrane. IP_3 binds to the IP_3 receptor on the endoplasmic reticulum and opens Ca^{2+} channels. The PLC blocker almost completely abolished the relaxation and the endothelial [Ca^{2+}] rise in response to carbachol. This suggests that the release from the intracellular stores is needed for Ca^{2+} entry, which is presumably responsible for the sustained carbachol induced rise in $[Ca^{2+}]_i$ observed in the presence of extracellular Ca^{2+}, and which probably occurs via store operated Ca^{2+} channels. This is supported by others' findings which have shown the importance of store release of Ca^{2+} in the EDHF response and even that the response could be mimicked by using an agonist that empties the Ca^{2+} store (Edwards et al., 2008). This was shown to be due to the Ca^{2+} release leading to hyperpolarisation of the endothelial cell via IK_{Ca} and SK_{Ca} channel activation (Fukao et al., 2005).

The gap junction inhibitor 18α-GA almost totally blocked the relaxation to carbachol, supporting a role for MEGJs in the EDHF response that has been found in other tissues such as the rat mesenteric artery, in which 18α-GA abolished the response to

acetylcholine (Jin 2008). In the rat cremaster arterioles it has been previously shown that electrical coupling through MEGJs was vital in the EDHF response as this was blocked using gap junction inhibitors (McSherry 2006). However in mouse cremaster electrical coupling has shown to be weak and not important for the EDHF response (Siegl 2005). This probably represents a difference between species, as the expression of MEGJ proteins varies between tissues (de Wit 2010). During the experiments, the arterioles were strongly pre-constricted with phenylephrine so were under a large level of excitation. The efficacy of 18α-GA in blocking the carbachol response is consistent with the finding that MEGJs are the predominant pathway when VSM are highly stimulated (Dora 2008). This has been proposed to be due to the opening of BK_{Ca} channels on the VSM due to the rise in $[Ca^{2+}]_i$ associated with a high level of stimulation, leading to K^+ efflux. This would increase the K^+ concentration in the myoendothelial space meaning that the K^+ released via the SK_{Ca} and IK_{Ca} channels on the endothelium during the EDHF response is swamped by the 'K^+ cloud' (Edwards & Weston, 2004). This stops the activation of the Na^+/K^+ ATPase on the VSM so K^+ cannot act as an EDHF (Mather et al., 2005), resulting in MEGJs becoming the dominant mechanism in this state, and could explain differences in the findings between some studies.

The selective IK_{Ca} and SK_{Ca} channel opener NS309 was used to mimic the EDHF response. It has been shown by others to open the IK_{Ca} and SK_{Ca} channels, leading to hyperpolarisation of the endothelial cell and relaxation of the smooth muscle (Leuranguer et al., 2008). It caused a significant vasodilatation in the rat cremaster arterioles, demonstrating the importance of this pathway in these vessels. 18α-GA was used to block the MEGJs and this was found to almost abolish the relaxation to

NS309. This supports the previous finding that shows the EDHF response is working through the MEGJs.

2.4.2 Summary

These results show that in the rat cremaster arterioles, the EDHF relaxation pathway is the dominant mechanism in response to carbachol. The EDHF pathway is primarily mediated through the SK_{Ca} and IK_{Ca} channels, and is dependent on MEGJs to cause relaxation in the smooth muscle, at least under the conditions used in these experiments. Ca^{2+} release from intracellular stores in the endothelial cell was also found to be vital for the EDHF response.

Chapter 4: The role of ROS in the EDHF response.

4.1 Introduction

4.1.1 ROS in the EDHF response

ROS, in particular H_2O_2, have been suggested to be involved in the EDHF response (Faraci 2006). Studies have shown that H_2O_2 can act as an endothelium dependent diffusible factor acting on the smooth muscle directly to cause hyperpolarisation and relaxation (Rojas 2006). However, it also appears that H_2O_2 may act on the endothelial cell itself to promote the EDHF response, possibly by increasing intracellular Ca^{2+} (Edwards 2008). I therefore carried out experiments designed to explore how ROS were involved in the EDHF response to carbachol.

To investigate the dependency on ROS during the relaxation, I used the antioxidants SOD and catalase. SOD dismutates superoxide into H_2O_2, and catalase breaks down H_2O_2 to water (Halliwell and Gutteridge, 2007). These antioxidants have been used to scavenge ROS in other studies and using ROS sensitive fluorescent indicators they have been shown to reduce ROS levels (e.g. Samora et al., 2008). H_2O_2 can be converted into the reactive hydroxyl radical by the Fenton reaction. This reaction requires the presence of iron (Kayyali et al.,1998). Iron chelators were used to block this reaction to investigate whether the hydroxyl radical was involved in the EDHF response. Desferrioxamine has been used widely as an iron chelator and was used in these experiments, but desferrioxamine can also scavenge ROS directly (Suematsu et al., 1993). For this reason, I used the specific iron chelators CP85 and CP94 which do not scavenge ROS (Rai et al., 1998). The hydroxyl radical can cause lipid peroxidation which can affect cell signalling and protein function (Smith et al., 1994). Vitamin E or α-tocopherol is a lipid soluble antioxidant which can scavenge ROS in the cell membrane (Mabile et al., 1995). This was used to prevent lipid peroxidation

to see if this is involved in the EDHF response. To investigate whether ROS are acting on the intracellular Ca^{2+} release, a Ca^{2+} free preparation was used. This prevented extracellular Ca^{2+} influx while maintaining the release from the Ca^{2+} stores (Hu et al., 1998).

4.1.2 Source of ROS

ROS can be produced by a number of sources as part of normal physiological processes (Takaki et al., 2008). To confirm that ROS generation was being blocked, ideally measuring ROS levels during the response would have been carried out while using different inhibitors. However, a number of different fluorescent ROS indicators and protocols were tested, but it was not possible to successfully load the cells with the ROS indicator, as preliminary experiments failed to produce any controls or reliable readings. This could be because the preparation is small or that the tissue is in situ, while many protocols are based on use in cultured cells (Suematsu et al., 1993). The interpretation of observations arising from commonly used ROS indicators is also controversial (Dunstan et al., 2000; Wardman et al., 2007). As an alternative, I investigated the source of ROS in the rat cremaster arterioles by examining the effects of inhibitors of different enzymes and processes that are known to generate ROS on the EDHF response to carbachol. Antioxidants were also used to show the involvement of ROS during the EDHF response. The different mechanisms involved in ROS production are described in the general introduction.

4.1.3 EETs

EETs are produced by the metabolism of arachidonic acid by cytochrome P450, in the same pathway that can produce ROS (Fleming et al., 2001). They have been suggested to be a potential EDHF in some tissues (Fleming et al., 2004; Hercule et al., 2009). The role of EETs in the EDHF response in rat cremaster arterioles was investigated in several ways. Exogenous EETs were applied to study the direct effect on vascular tone and endothelial Ca^{2+}. The role of EETs during the carbachol response was studied by blocking EET breakdown by soluble epoxide hydrolase (sEH) using the inhibitor AUDA (Campbell & Fleming, 2010). This would increase EETs concentration in the preparation. The EETs antagonist EEZE was also used to block any response that EETs may have during the EDHF response to carbachol (de Wit et al., 1999).

4.2 Methods

The role of ROS in the EDHF mediated response to carbachol was investigated using intravital microscopy and the tissue preparation and setup as described in the general methods chapter. Different antioxidants were used to scavenge ROS and to understand their role in the EDHF pathway and to identify the particular species of ROS involved. Selective inhibitors of the enzymes that are potentially involved in ROS production were used to investigate the source of the ROS in rat cremaster arterioles.

4.2.1 Protocol

The rat cremaster arterioles were pre-constricted with 30μM phenylephrine and the experiments took place in the presence of 300μM L-NAME and 3μM indomethacin to ensure only EDHF-mediated relaxation was present. 10μM carbachol was added to the preparation and the response was compared to the response in the presence of different antioxidants and inhibitors by measuring the relaxation and change in endothelial cell Ca^{2+}. The Ca^{2+} free experiments were carried out as previously described in Chapter 3. The pre-treatment time was 2 minutes for the antioxidants and inhibitors that were added other than 10 minutes for EEZE, AUDA, ATZ, AACOCF$_3$ allopurinol and Ro 31-8220, and 15 minutes for 18α-GA, NDGA, PPOH and 17-ODYA. 18α-GA and 17-ODYA were not fully reversible so experiments were performed at the end of the run.

4.2.2 Electrophysiology experiments

The EDHF response is characterised by a hyperpolarisation of the endothelial and smooth muscle cells leading to relaxation. To support the experiments involving intravital microscopy, electrophysiology experiments were carried out in Dr Kim Dora's lab at the University of Oxford. This allowed for the direct measurement of changes in the membrane potential of the smooth muscle cells of the artery.

An isolated perfused rat cremasteric artery was used for these experiments. 200-270g Wistar rats were used as the cremaster artery has to be dissected and mounted onto the micropipettes for perfusion and therefore must be larger than the arterioles used in the

intravital microscopy preparation (diameter ~200 microns). The rats were killed in accordance with Schedule 1 by overdose of the anaesthetic urethane. The cremaster muscle was then exposed as previously described in the General Methods. A second order artery with a diameter of around 200 microns was chosen and carefully dissected out and the surrounding connective tissue was removed. A length of about 5mm was then mounted onto cannulation pipettes to allow perfusion. A microscope was used to aid careful dissection and mounting of the artery. A Perspex plate was attached to the movable stage of a microscope allowing for visualisation of the artery. The cannulation pipettes were fixed and held by two micromanipulators. The arteries were continually superfused (4ml/min) with a heated solution of PSS at 37ºC. Intracellular recordings of the membrane potential were made using glass microelectrodes filled with 2M KCl (giving tip resistances of 90-150 MΩ), and a Neurolog amplifier system, and data were acquired using Clampex 8.0.2 software. The microelectrodes were used to carefully impale the smooth muscle cell. This was done manually using a third piezoelectric precision micromanipulator, which was placed on a stand to hold the microelectrodes at a 45º angle to impale smooth muscle cells above the midplane of the vessels. Successful recordings were characterised by an abrupt deflection of signal on impalement of cells and an approximate return to the pre-impalement values on removal of the microelectrode. Following successful impalement of the microelectrode drugs were then added to the superfusate to elicit changes in the resting membrane potential of the smooth muscle cell using the same protocol as used for the intravital microscopy setup. L-NAME and indomethacin were present throughout. The K^+ channel opener cromakalim (3µM) was added as a positive control to elicit hyperpolarisation to ensure the microelectrode was measuring the membrane potential of the smooth muscle cell.

4.2.3 Analysis

The results of the carbachol controls and the experiments with an inhibitor present were paired so comparison was made on the same arteriole. The % relaxation and % 360/380 ratio increase are displayed in graphs as the control paired with the test experiment with the different inhibitors present. The electrophysiology experiments were also paired showing the change in membrane potential to carbachol and then the change with antioxidants present. The means $\pm$ the standard errors were calculated and a two-tailed paired t-test was used to compare the results. The difference between the compared results was considered significant if the p value was less than 0.05, represented by a * on the graph (** $p<0.01$, *** $p<0.001$).

4.3 Results

4.3.1 Antioxidants

The simultaneous application of the anti-oxidant enzymes SOD and catalase (100U/ml each) significantly reduced the EDHF mediated relaxation following carbachol application from 75.0% $\pm$ 7.6 with carbachol alone to 36.8% $\pm$ 11.4 in the presence of the anti-oxidants (see Fig. 4.1). The EDHF-mediated relaxation in response to carbachol with SOD and catalase was less than 60% of the relaxation to carbachol alone. There was also a reduction in the rise in endothelial cell Ca^{2+} when ROS were scavenged. Carbachol alone caused a ratio increase of 19.1% $\pm$ 3.5 whereas with the addition of SOD and catalase the increase was only 5.5% $\pm$ 2.1. This shows that the anti-oxidants significantly reduced the Ca^{2+} rise in the endothelial cells in response to carbachol with the increase inhibited by almost 75% (see Fig. 4.2).

The electrophysiology experiments showed that following carbachol application there was a hyperpolarisation of the smooth muscle membrane, characteristic of an EDHF response (see Fig. 4.3). In the presence of the antioxidants SOD and catalase the membrane hyperpolarisation in response to carbachol was reduced by nearly 75% (see Fig. 4.4), consistent with the findings from the intravital microscopy experiments showing an inhibition of the EDHF response by scavenging ROS.

In the Ca^{2+} free preparation, the addition of SOD and catalase reduced the transient rise in Ca^{2+} from 20.5% $\pm$ 2.8 to 10.4% $\pm$ 1.5 (see Fig. 4.6). A typical trace demonstrates this inhibition in the Ca^{2+} rise is shown in Fig. 4.7. This result suggests

that ROS are important in the release of Ca^{2+} from internal stores and that this is a vital step in the EDHF response. The application of SOD did not affect the Ca^{2+} rise significantly with a 22.3% $\pm$ 1.8 increase compared to 22.8% $\pm$ 1.9 with carbachol alone (see fig 4.7). Catalase alone however did reduce the ratio increase with a rise of 10.6% $\pm$ 1.6 in the presence of catalase following carbachol application compared to 23.3% $\pm$ 2.1, showing a reduction of nearly 60% (see Fig. 4.8).

The IK_{Ca} and SK_{Ca} channel opener NS309 has previously been shown to mimic the EDHF response leading to relaxation. The presence of SOD and catalase had no effect on the NS309 induced relaxation (see Fig. 4.9 & 4.10). This suggests that ROS involved in the EDHF pathway might be important upstream of the opening of the IK_{Ca} and SK_{Ca} channels.

Vitamin E or α-tocopherol is a fat soluble antioxidant that in particular targets lipid peroxidation in cell membranes. Treatment of the arterioles with α-tocopherol had no effect on the EDHF response to carbachol with no inhibition of relaxation or effect on the Ca^{2+} rise (see Fig. 4.11 & 4.12).

To further study the importance of ROS in the EDHF response I determined whether increasing ROS had any effect. Atz is a catalase inhibitor which will lead to an increase in H_2O_2. Pre-treatment with Atz had no effect on its own but when carbachol was added the EDHF response was enhanced with an increase in relaxation and a larger rise in endothelial cell Ca^{2+} (see Fig. 4.13 & 4.14). This suggests that increasing H_2O_2 enhances the EDHF response, at least in part by increasing endothelial cell Ca^{2+}.

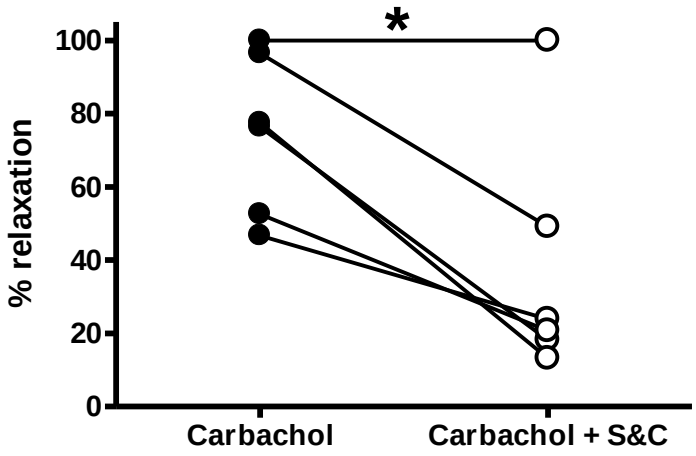

Figure 4.1. Effect of antioxidants on relaxation to carbachol.

The % relaxation to carbachol (75.0 % ± 7.6) was inhibited by the addition of the antioxidants SOD and catalase (100U/ml each) (36.8 % ± 11.4). Results show mean ± SE, n= 7, * $p < 0.05$.

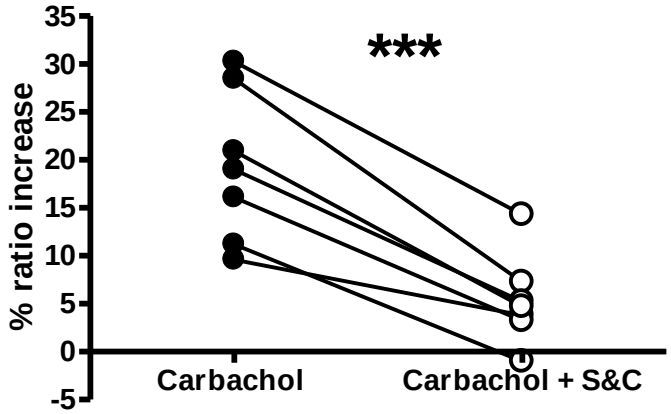

Figure 4.2. Effect of antioxidants on the ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (19.4 % ± 3.0) was inhibited by the presence of the antioxidants SOD and catalase (100U/ml each) (5.3 % ± 1.8). Results show mean ± SE, n=7 , *** $p < 0.001$.

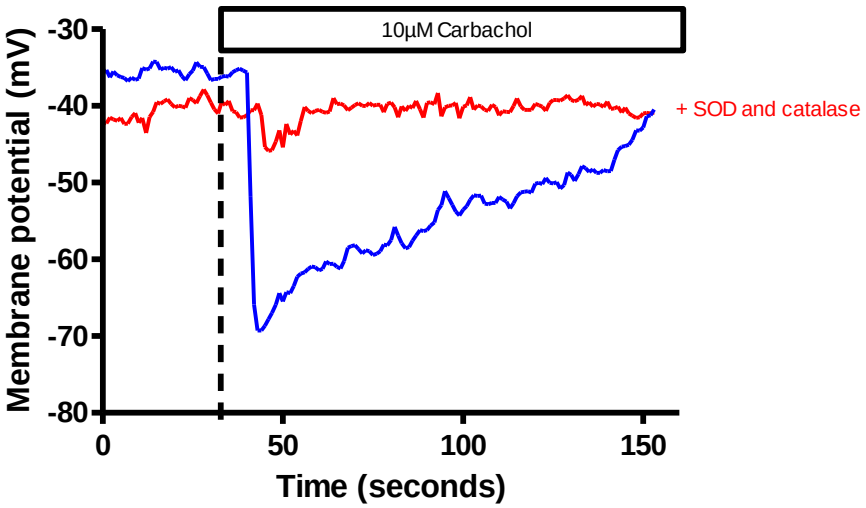

Figure 4.3. A typical trace showing the change in membrane potential in a smooth muscle cell in rat cremaster arteriole.

The hyperpolarisation to carbachol was reduced when the preparation was treated with the antioxidants SOD and catalase.

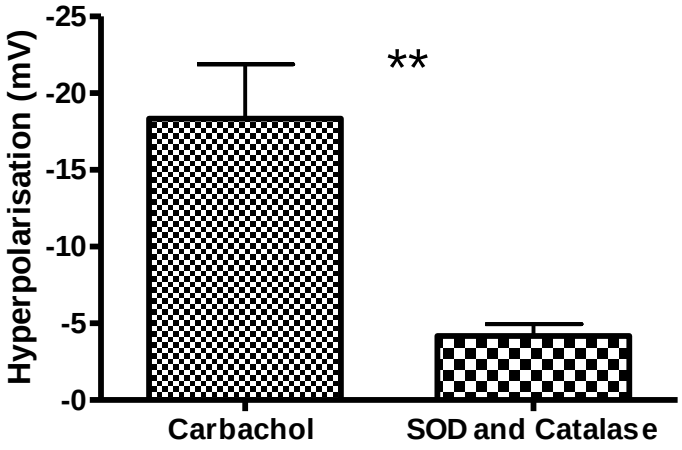

Figure 4.4. The effect of antioxidants on the change in smooth muscle membrane potential in response to carbachol.

The hyperpolarisation (change in mV from resting membrane potential) to carbachol (18.5 % $\pm$ 3.5) was inhibited by treatment with the antioxidants SOD and catalase (100U/ml each) (4.2 % $\pm$ 0.8). Results show mean $\pm$ SE, n=5 , ** $p < 0.01$.

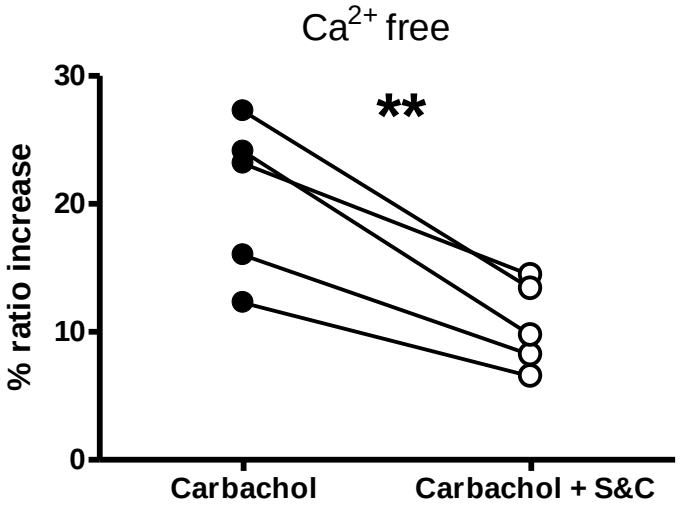

Figure 4.5. The effect of antioxidants on the ratio increase to carbachol in the Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (20.5 % ± 2.8) was attenuated by the antioxidants SOD and catalase (100U/ml each) (10.4 % ± 1.5). Results show mean ± SE, n=5, ** $p < 0.01$.

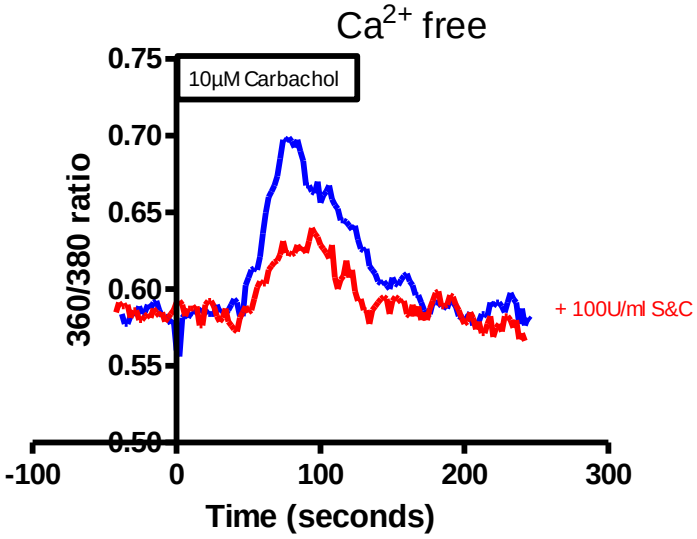

Figure 4.6. Typical trace of the increase in 360/380nm ratio in response to carbachol in a Ca^{2+} free preparation.

The addition of the antioxidants SOD and catalase inhibited the release of Ca^{2+} from the intracellular stores.

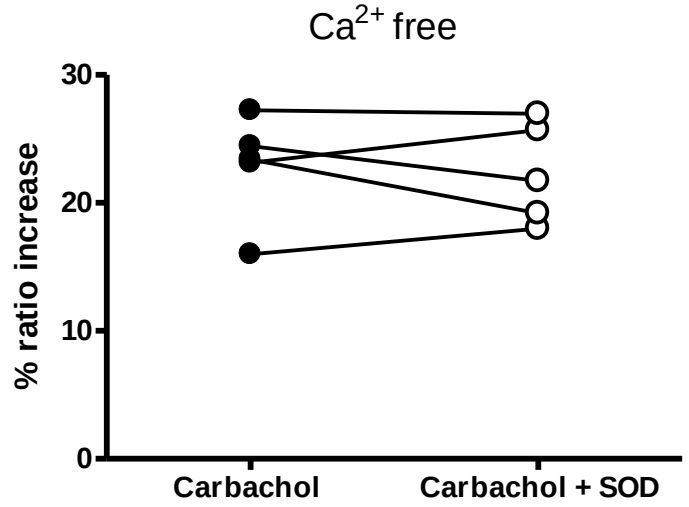

Figure 4.7. The effect of SOD on the ratio increase to carbachol in the Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (22.8 % ± 1.9) was not significantly affected by the antioxidant SOD (100U/ml) (22.3 % ± 1.8). Results show mean ± SE, n=5, (n.s).

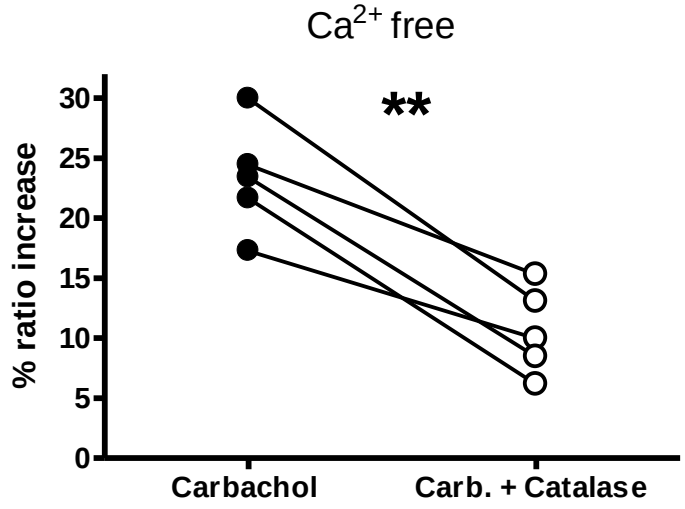

Figure 4.8. The effect of catalase on the ratio increase to carbachol in the Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (23.3 % ± 2.1) was inhibited by the antioxidant catalase (100U/ml) (10.6 % ± 1.6). Results show mean ± SE, n=5, ** $p < 0.01$.

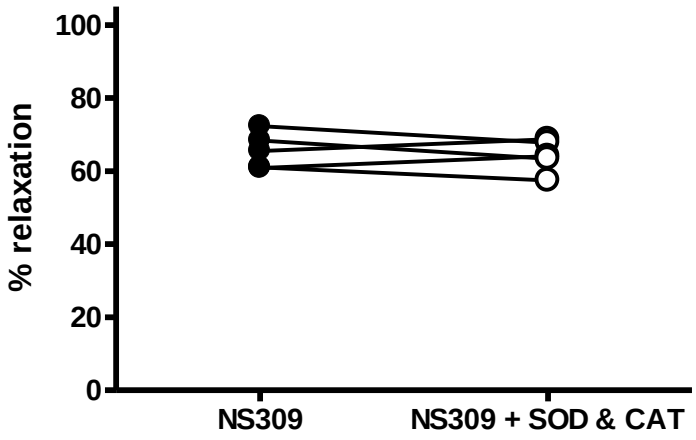

Figure 4.9. Antioxidants did not affect NS309 induced relaxation.

The relaxation to the SK_{Ca} and IK_{Ca} channel opener NS309 (10µM) (65.6 % ± 2.2) was not significantly affected by the antioxidants SOD and catalase (100U/ml each) (64.3 % ± 2.0). Results show mean ± SE, n=5, (n.s).

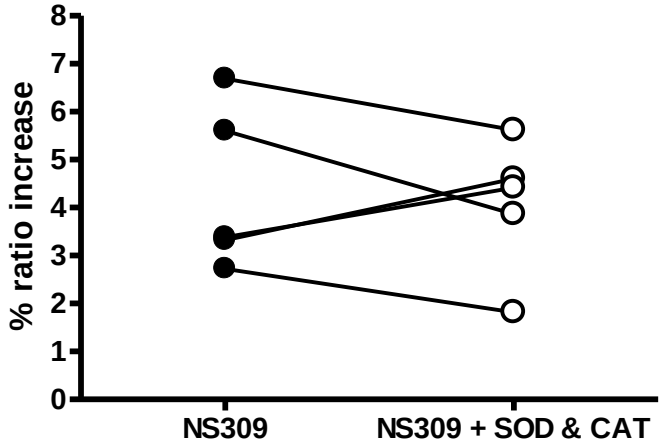

Figure 4.10. Antioxidants did not affect the 360/380nm ratio increase to NS309.

The 360/380nm ratio increase to NS309 (10µM) (4.3 % ± 0.8), was not significantly altered by the antioxidants SOD and catalase (100U/ml each) (4.1 % ± 0.6). Results show mean ± SE, n=5, (n.s).

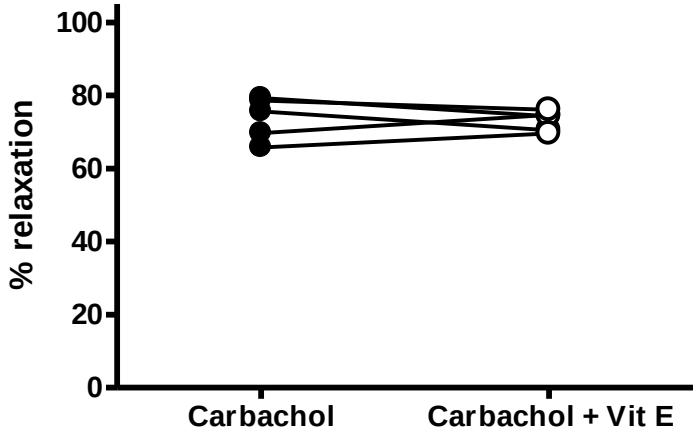

Figure 4.11. Vitamin E did not affect the relaxation to carbachol.

The relaxation to carbachol (73.8 % ± 2.6) was not significantly affected by the lipid soluble antioxidant vitamin E (100μM) (73.0 % ± 1.3). Results show mean ± SE, n=5, (n.s).

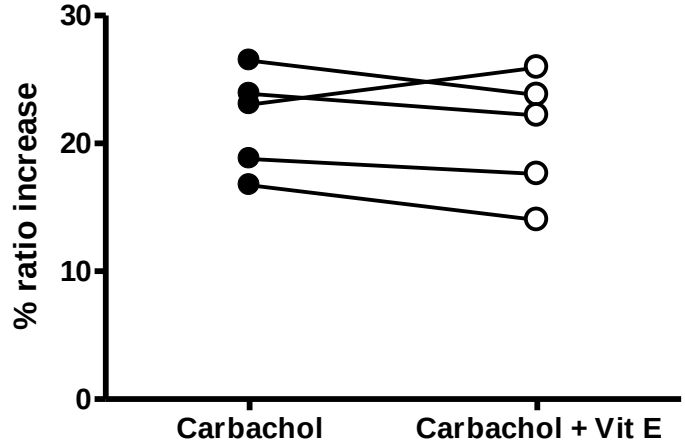

Figure 4.12. Vitamin E did not affect the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (21.8 % ± 1.8) was not changed by the lipid soluble antioxidant vitamin E (100μM) (20.7 % ± 2.2). Results show mean ± SE, n=5, (n.s).

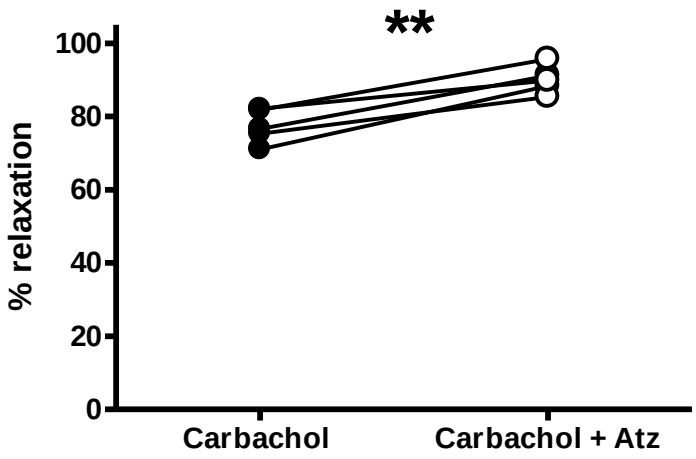

Figure 4.13. Catalase inhibition potentiated the relaxation to carbachol.

The relaxation to carbachol (77.4 % ± 2.1) was increased by the catalase blocker Atz (50mM) (90.1 % ± 1.7). Results show mean ± SE, n=5, ** $p < 0.01$.

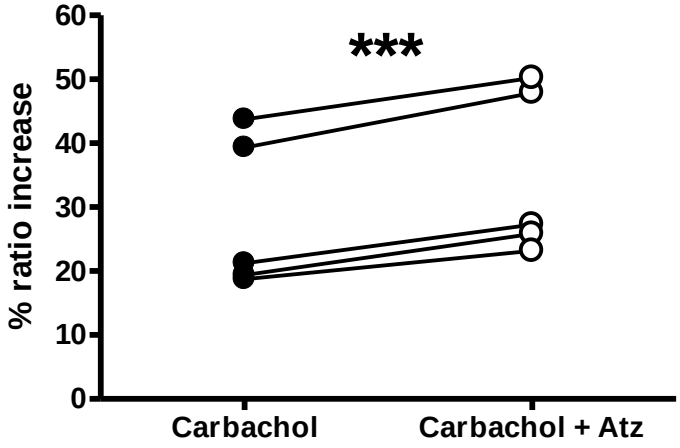

Figure 4.14. Catalase inhibition potentiated the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (28.5 % ± 5.4) was enhanced by the catalase blocker Atz (50mM) (34.9 % ± 5.8). Results show mean ± SE, n=5, *** $p < 0.001$.

4.3.2 Iron Chelators

Application of the iron chelator desferrioxamine (DFO; 100μM) significantly reduced the relaxation in response to carbachol from 64.5% ± 15.0 to 20.6% ± 3.5 (see Fig. 4.15). The addition of DFO also caused a significant reduction in the Ca^{2+} increase during the EDHF response from 31.7% ± 11.4 with carbachol alone to only 9.4% ± 6.5 with DFO present (see Fig. 4.16). However, DFO also scavenges free radicals directly, so the effects of the hydroxypyridine iron chelators CP85 and CP94, which do not directly scavenge free radicals, were also tested. The cell impermeant CP85 (100μM) reduced the relaxation from 77.8% ± 7.5 with carbachol alone to 44.0% ± 5.3 in the presence of CP85 (see Fig. 4.17), and inhibited the recorded rise in Ca^{2+} significantly from 20.2% ± 3.5 to 11.3% ± 2.8 (see Fig. 4.18). The cell permeant CP94 (100μM) also significantly reduced the EDHF mediated relaxation to 38.7% ± 7.7 (see Fig. 4.19). The Ca^{2+} rise in the presence of CP94 was less than 30% of the rise in response to carbachol alone (see Fig. 4.20.). The ratio increase was reduced from 20.2% ± 3.5 to 5.5% ± 0.9.

In the Ca^{2+} free preparation the cell impermeant CP85 did not significantly reduce the Ca^{2+} increase (18.5% ± 2.7) compared to carbachol alone (18.8% ± 1.3) (see Fig. 4.21). On the other hand, the cell permeant CP94 did cause significant reduction in the Ca^{2+} increase in comparison to carbachol alone with a fall from 18.0% ± 3.7 to only 3.4% ± 1.2 (see Fig. 4.22). These data indicate that, unlike CP85, CP94 inhibits Ca^{2+} release from stores.

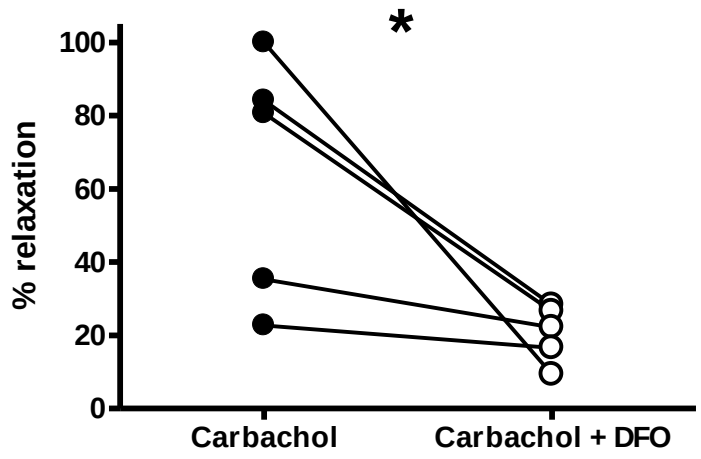

Figure 4.15. Relaxation to carbachol in the presence of DFO.

The iron chelator DFO (100μM) inhibited the relaxation to carbachol from 64.5 % ± 15.0 to 20.6 % ± 3.5. Results show mean ± SE, n= 5, * $p < 0.05$.

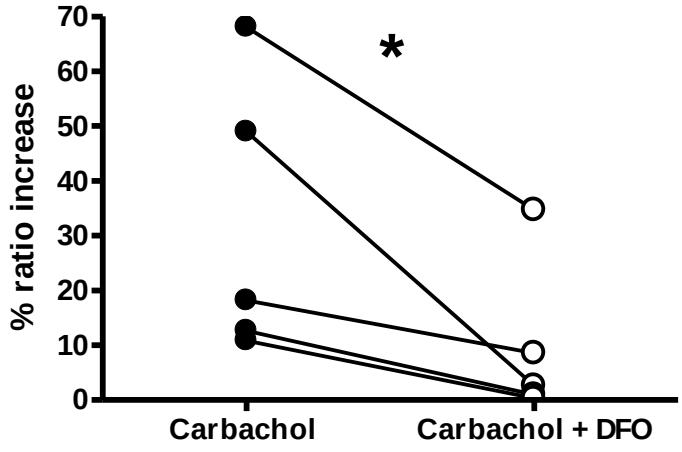

Figure 4.16. The effect of DFO on the 360/380nm ratio increase in response to carbachol.

The 360/380nm ratio increase to carbachol (31.7% ± 11.4) was inhibited by the iron chelator DFO (100μM) (9.4 % ± 6.5). Results show mean ± SE, n=5 , * $p < 0.05$.

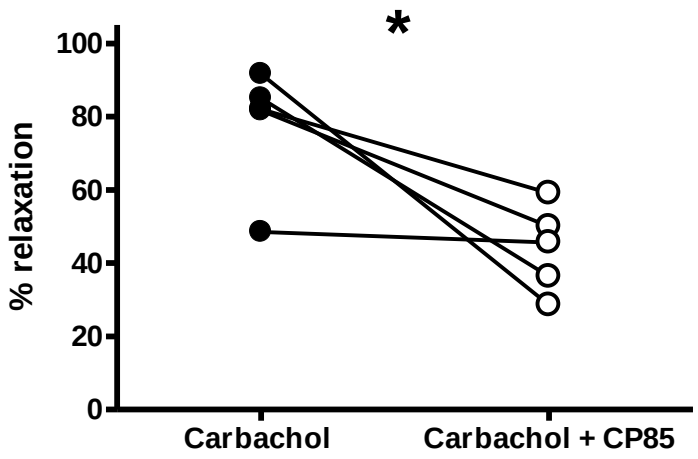

Figure 4.17. The effect of the iron chelator CP85 on carbachol relaxation.

The % relaxation to carbachol (77.8 % ± 7.5) was inhibited by the cell impermeant iron chelator CP85 (100μM) (43.9 % ± 5.3). Results show mean ± SE, n=5 , * $p < 0.05$.

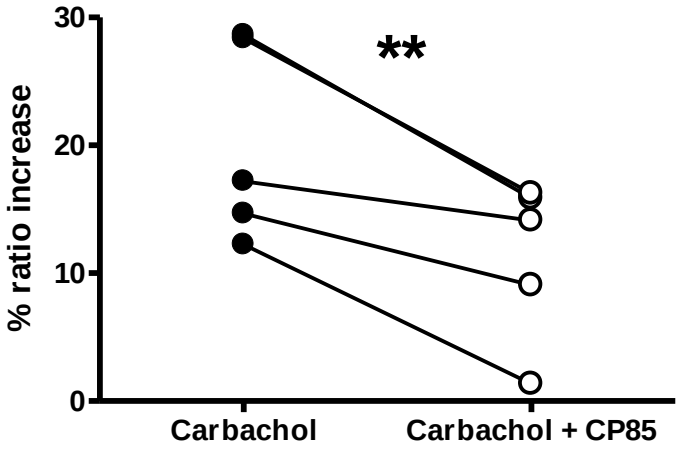

Figure 4.18. The effect of CP85 on the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (20.2 % ± 3.5) was inhibited by the cell impermeant iron chelator CP85 (100μM) (11.3 % ± 2.8). Results show mean ± SE, n=5 , ** $p < 0.01$.

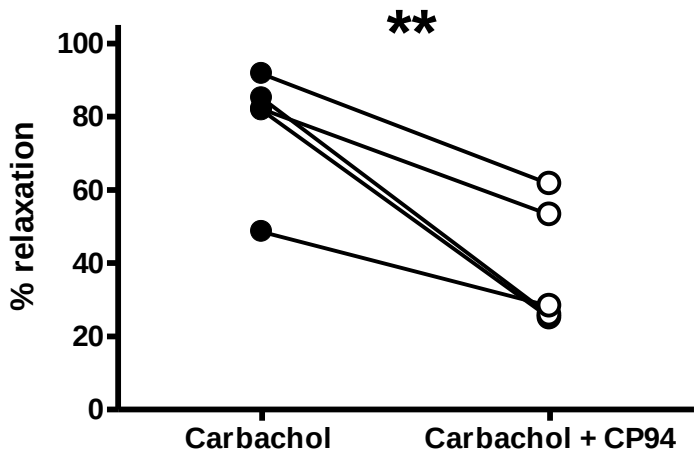

Figure 4.19. Effect of iron chelation with CP94 on the relaxation to carbachol.

The % relaxation to carbachol (77.7 % ± 7.5) was attenuated by the cell permeant iron chelator CP94 (100μM) (38.7 % ± 7.2). Results show mean ± SE, n=5, ** $p < 0.01$.

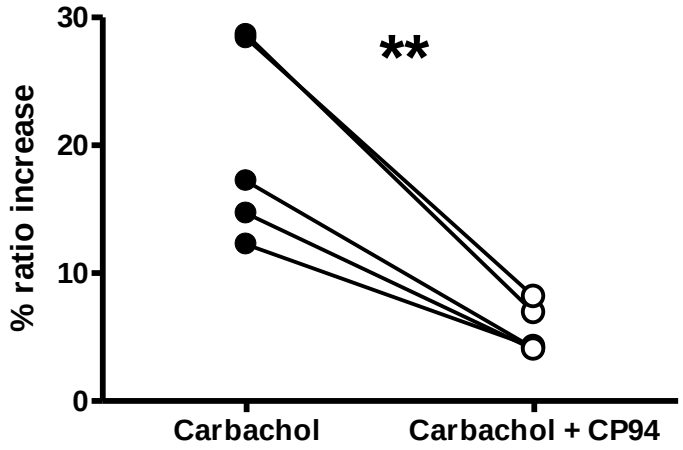

Figure 4.20. Effect of iron chelation with CP94 on the ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (20.2 % ± 3.5) was inhibited by the cell permeant iron chelator CP94 (100μM) (5.5 % ± 0.9). Results show mean ± SE, n=5, ** $p < 0.01$.

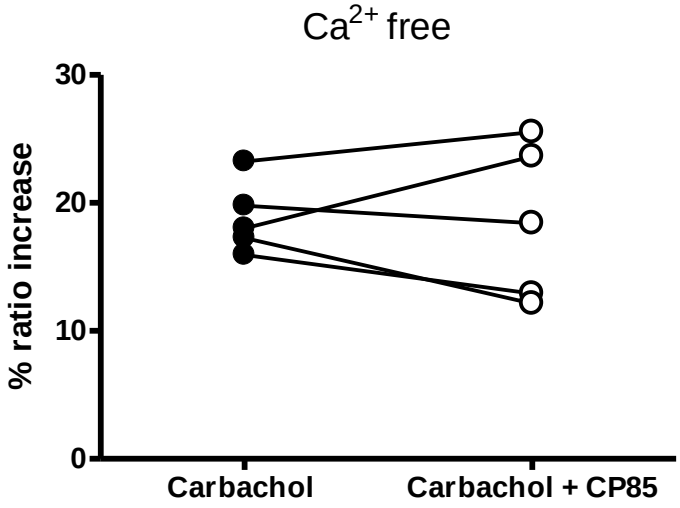

Figure 4.21. The effect of the cell impermeant iron chelator CP85 on the ratio increase to carbachol in a Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (18.8 % ± 1.3) was not affected by CP85 (100μM) (18.5 % ± 2.7). Results show mean ± SE, n=5 , (n.s).

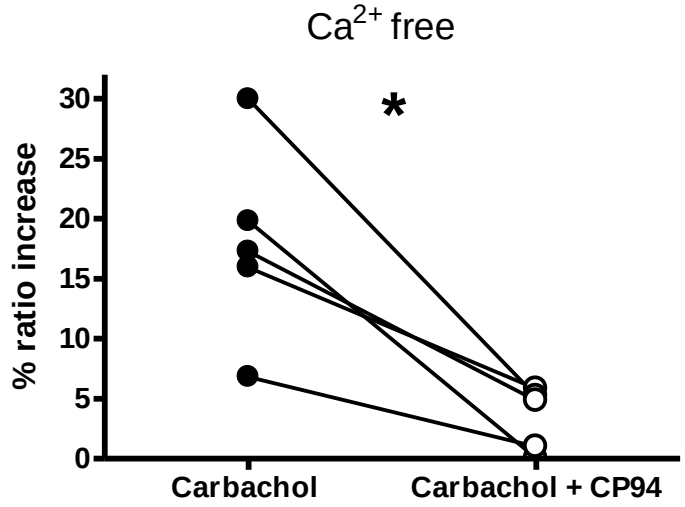

Figure 4.22. The effect of the cell permeant iron chelator CP94 on the ratio increase to carbachol in the Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (18.0 % ± 3.7) was inhibited by the iron chelator CP94 (100μM) (3.4 % ± 1.2). Results show mean ± SE, n=5 , * $p < 0.05$.

4.3.3 ROS producing enzymes

I used a range of inhibitors to investigate the role of the various pathways that can lead to cellular ROS production in the response to carbachol.

Lipoxgenase (LOX) is a potential source of ROS, and can be blocked with the drug NDGA. I found that NDGA had no effect on the EDHF response to carbachol with no reduction in relaxation or endothelial cell Ca^{2+} (see Fig. 4.23 & 4.24), indicating that in rat cremaster arterioles that LOX is not a major source of ROS.

Mitochondria are known to be a major source of ROS, through their production in the electron transport chain. Rotenone is a poison that inhibits the transfer of electrons to complex I, thus blocking ROS production at both complexes 1 and 3. In these experiments rotenone had no effect on the response to carbachol (see Fig. 4.25 & 4.26). This suggests that mitochondrial production of ROS is not an important part of the EDHF mechanism in this tissue.

The enzyme xanthine oxidase can produce ROS, in particular superoxide. Allopurinol is a xanthine oxidase inhibitor. Pre-treatment with allopurinol was found to have no effect on the EDHF response to carbachol (see Fig. 4.27 & 4.28). This pathway therefore does not appear to be involved in the EDHF mechanism in these arterioles.

The NADPH oxidase enzymes have been shown to be an important source of ROS in different tissues and have been implicated in the EDHF response. Apocynin is an inhibitor of NADPH oxidase so was used to study their role in this tissue bed. Pre-

treatment with apocynin had no effect on the EDHF response to carbachol (see Fig. 4.29 & 4.30). Protein kinase C (PKC) is an enzyme which is important in signal transduction and is involved in increasing NADPH oxidase activation. The PKC inhibitor Ro 31-8220 had no effect on the EDHF response (see Fig. 4.31 & 4.32). This suggests that although NADPH oxidase is an important source of ROS, in rat cremaster arterioles this pathway is not important for the EDHF response, at least under basal conditions (see below).

The cytochrome P450 enzymes are a large family of enzymes that are present in many tissues and involved in the metabolism of many endogenous and exogenous compounds. They have also been shown to be a source of ROS in some tissues. To investigate this, different CYP450 inhibitors were used. PPOH and 17-ODYA are both non-specific CYP450 blockers that work through different mechanisms. Both blockers were found to significantly inhibit the EDHF response to carbachol with a reduction in both the relaxation and the endothelial cell [Ca^{2+}] rise (see Fig. 4.33, 4.34. 4.35 & 4.36). To investigate this further, the experiments were carried out in the Ca^{2+} free preparation. The non-specific CYP450 blockers were also found here to inhibit the transient rise in Ca^{2+} in response to carbachol (see Fig. 4.40 & 4.41). To narrow down the member of the CYP450 family which is involved, a more specific inhibitor was used. Previous work has implicated CYP2C9 as the isoform involved in ROS production, and it has been shown in endothelial cells. Sulfaphenazole is a specific CYP2C9 inhibitor and it was found to inhibit the EDHF response to carbachol (see 4.37 & 4.38). A typical trace shows this inhibition (see Fig. 4.39). In the Ca^{2+} free preparation sulfaphenazole also inhibited the transient increase in Ca^{2+} in response to carbachol (see Fig. 4.42). A typical trace showing the inhibition by

sulfaphenazole of the transient rise in Ca^{2+} from store release is shown in Fig. 4.43. These results suggest that CYP2C9 is important in the EDHF pathway, possibly as a source of ROS.

To further investigate the role of CYP2C9 in producing ROS, upstream mechanisms were studied. Arachidonic acid is a fatty acid that is released from the phospholipid membrane by phospholipase A_2 (PLA_2). It is metabolised by CYP2C9 and ROS are produced during this process. To study the involvement of this pathway the PLA_2 inhibitor $AACOCF_3$ was used. $AACOCF_3$ was found to significantly inhibit the EDHF response to carbachol (see Fig. 4.44 & 4.45). This suggests PLA_2 is an important part of the EDHF pathway, possibly as a source of arachidonic acid which is metabolised by CYP2C9 producing ROS.

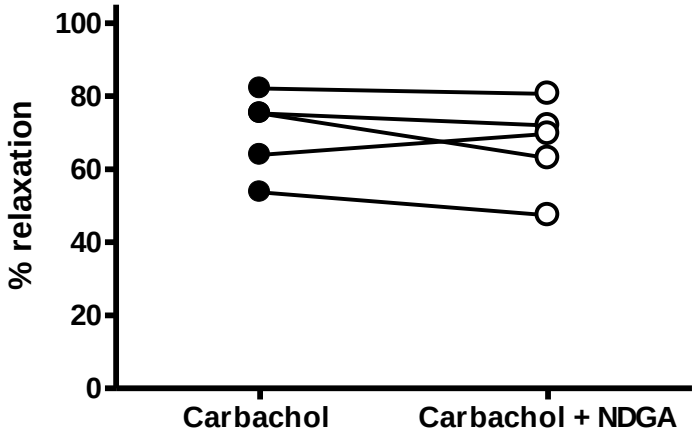

Figure 4.23. The effect of LOX inhibition on the relaxation to carbachol.

The relaxation to carbachol control (70.1 % ± 5.0) was not affected by the lipoxgenase inhibitor NDGA (50µM) (66.6 % ± 5.6). Results show mean ± SE, n=5, (n.s)

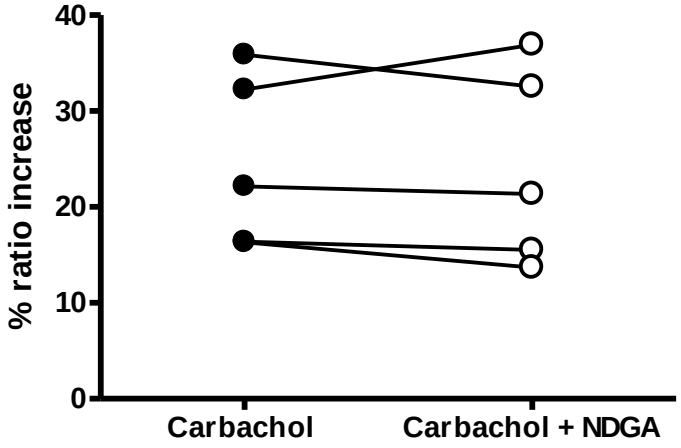

Figure 4.24. The effect of LOX inhibition on the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (24.6 % ± 4.1) was not significantly affected by the lipoxgenase blocker NDGA (50µM) (24.0 % ± 4.6). Results show mean ± SE, n=5, (n.s)

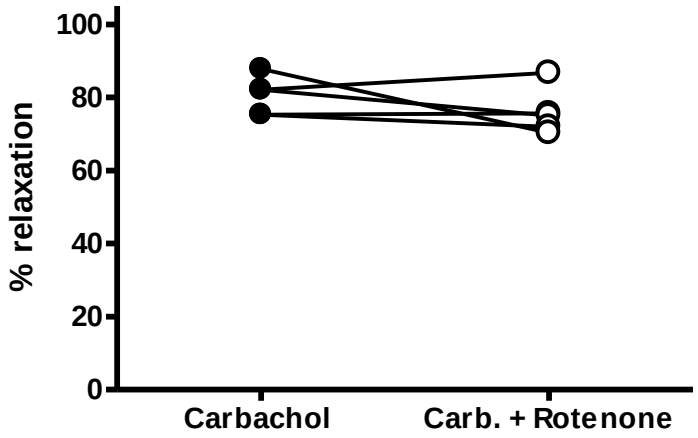

Figure 4.25. The role of mitochondrial ROS production in the relaxation to carbachol.

The relaxation to carbachol (80.5 % ± 2.4) was not affected by the complex I inhibitor rotenone (10µM) (76.0 % ± 2.9). Results show mean ± SE, n=5, (n.s)

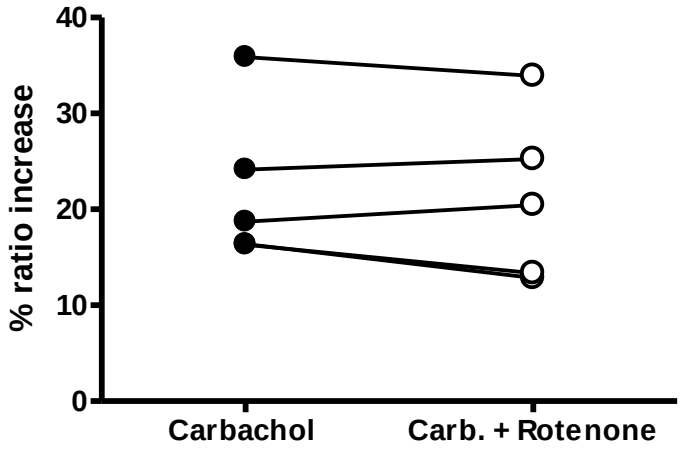

Figure 4.26. The role of mitochondrial ROS production in the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (22.3 % ± 3.7) was not significantly affected by the complex I blocker rotenone (10µM) (21.2 % ± 3.9). Results show mean ± SE, n=5, (n.s)

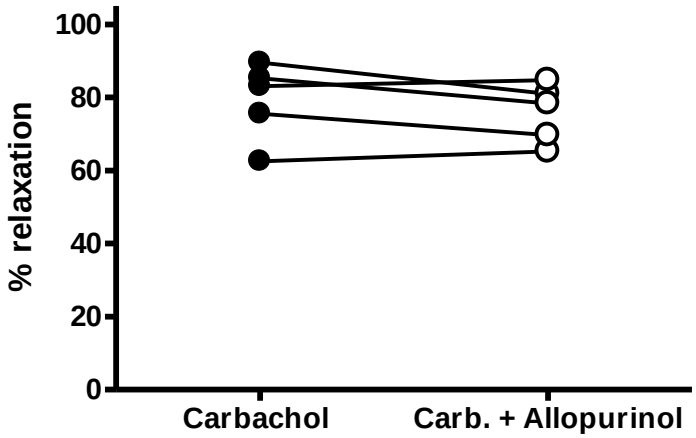

Figure 4.27. The effect of xanthine oxidase inhibition with allopurinol on relaxation to carbachol.

The % relaxation to carbachol (79.2 % ± 4.7) was not changed by the xanthine oxidase inhibitor allopurinol (100μM) (75.8 % ± 3.6). Results show mean ± SE, n=5, (n.s)

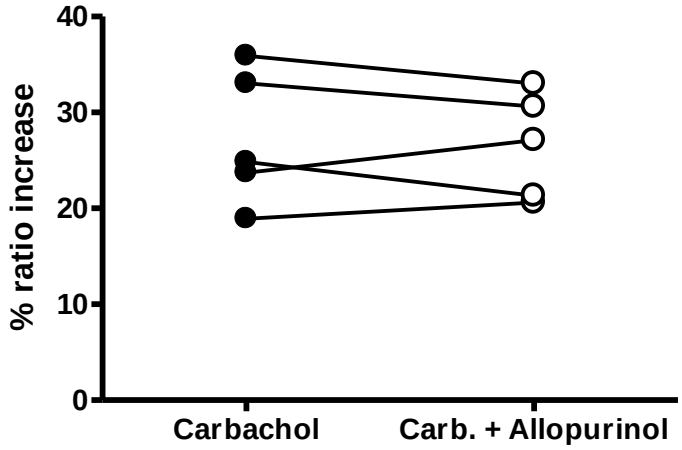

Figure 4.28. The effect of xanthine oxidase inhibition with allopurinol on the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (27.3 % ± 3.1) was unaffected by the xanthine oxidase inhibitor allopurinol (100μM) (26.5 % ± 2.5). Results show mean ± SE, n=5, (n.s)

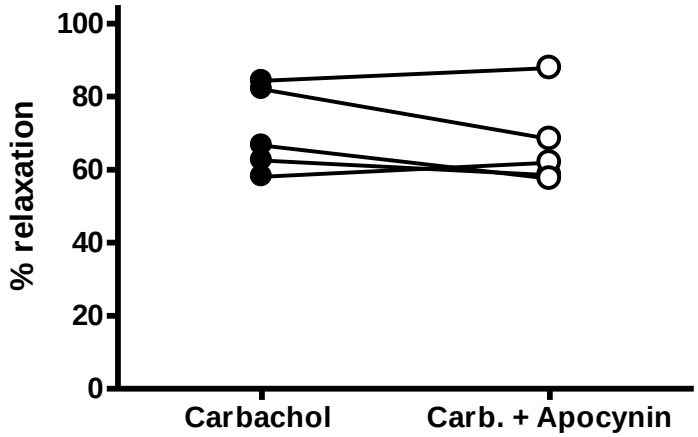

Figure 4.29. The role of NADPH oxidase in the relaxation to carbachol.

The relaxation to carbachol (70.7 % ± 5.3) was not significantly changed by the presence of the NADPH oxidase blocker apocynin (1µM) (66.8 % ± 5.6). Results show mean ± SE, n=5, (n.s)

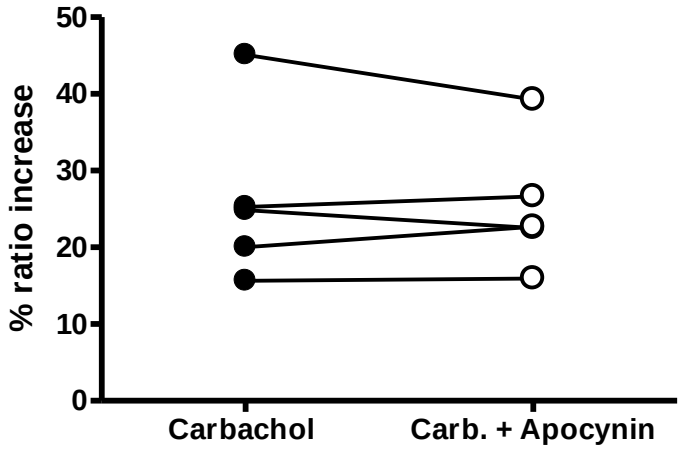

Figure 4.30. The role of NADPH oxidase in the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (26.2 % ± 5.0) was not affected by NADPH oxidase inhibition by apocynin (1µM) (25.4 % ± 3.9). Results show mean ± SE, n=5, (n.s)

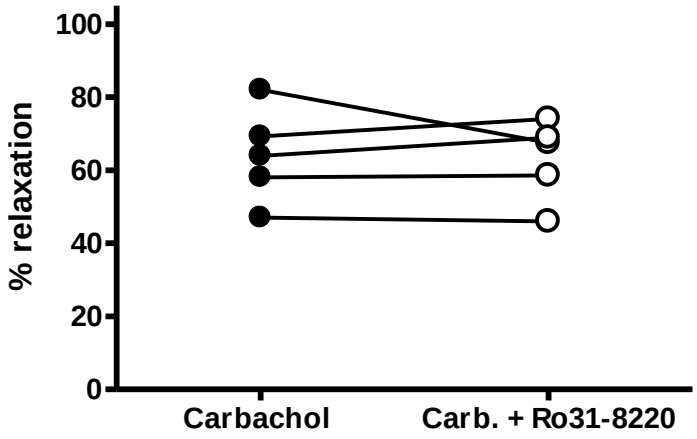

Figure 4.31. The role of PKC in the relaxation to carbachol.

The relaxation to carbachol (64.1 % ± 5.8) was not significantly affected by the PKC blocker Ro31-8220 (3µM) (63.0 % ± 4.9). Results show mean ± SE, n=5, (n.s).

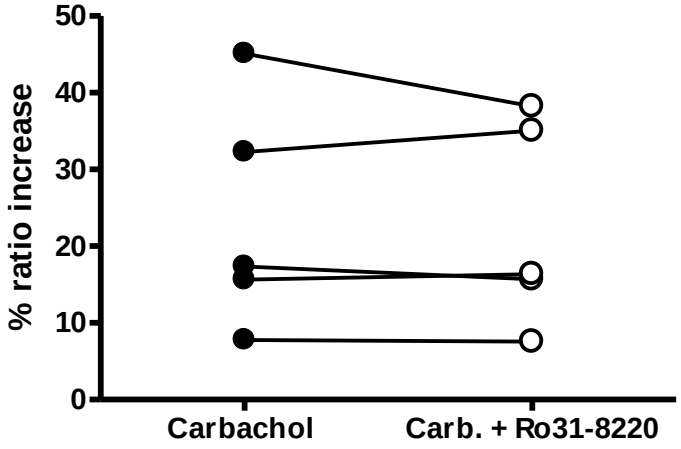

Figure 4.32. The role of PKC in the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (23.6 % ± 6.7) was not changed by the PKC inhibitor Ro31-8220 (3µM) (22.6 % ± 6.0). Results show mean ± SE, n=5, (n.s).

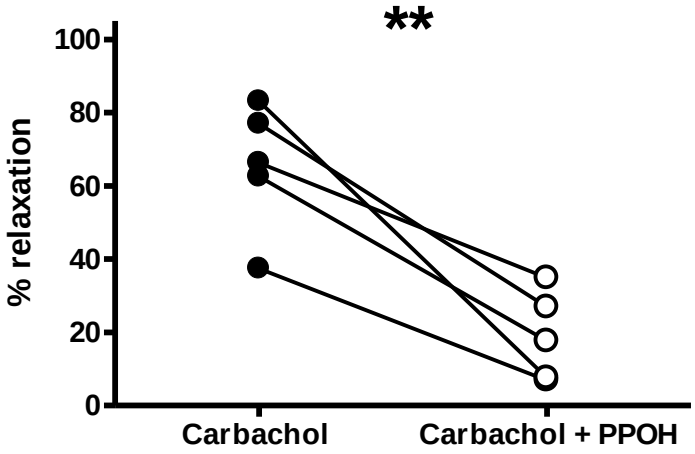

Figure 4.33. The effect of CYP450 inhibition on carbachol relaxation.

The % relaxation to carbachol (65.3 % ± 7.9) was inhibited by the CYP450 blocker PPOH (10μM) (18.8 % ± 5.5). Results show mean ± SE, n=5, ** $p < 0.01$.

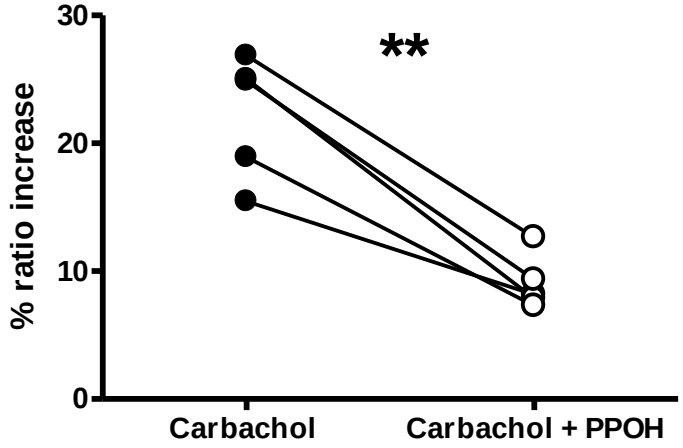

Figure 4.34. The effect of CYP450 inhibition on the 360/380 nm ratio increase to carbachol.

The CYP450 blocker PPOH (10μM) attenuated the 360/380nm ratio increase to carbachol from 22.2 % ± 2.2 to 9.1 % ± 1.0). Results show mean ± SE, n=5, ** $p < 0.01$.

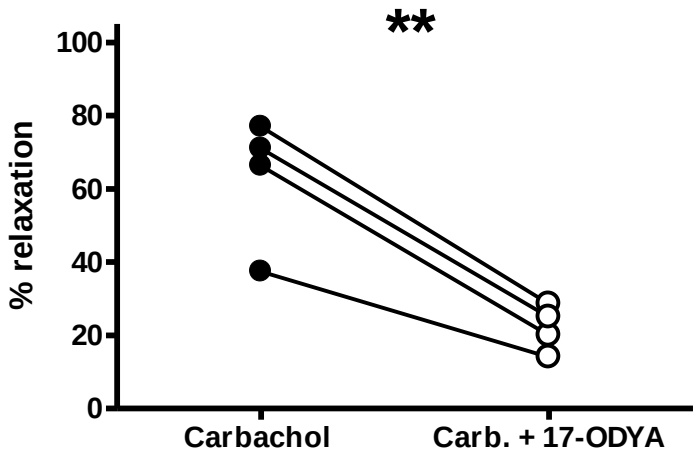

Figure 4.35. The effect of CYP450 inhibition on the relaxation to carbachol.

The % relaxation to carbachol (62.9 % ± 8.8) was inhibited by the CYP450 blocker 17-ODYA (50μM) (21.9 % ± 3.2). Results show mean ± SE, n=4 , ** $p < 0.01$.

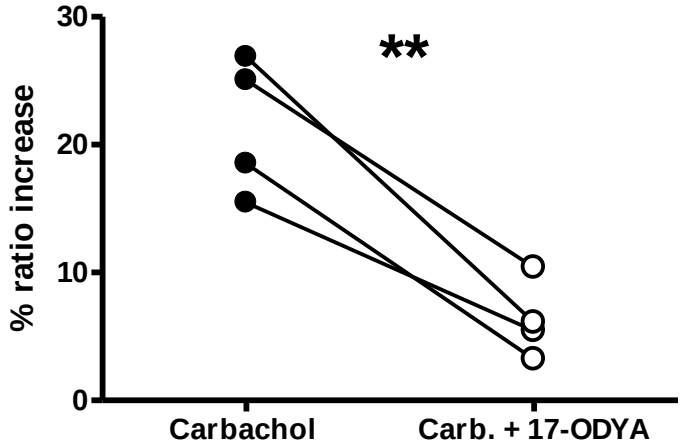

Figure 4.36. The effect of CYP450 inhibition on the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (21.5 % ± 2.7) was inhibited by the CYP450 blocker 17-ODYA (50μM) (6.3 % ± 1.5). Results show mean ± SE, n=4 , ** $p < 0.01$.

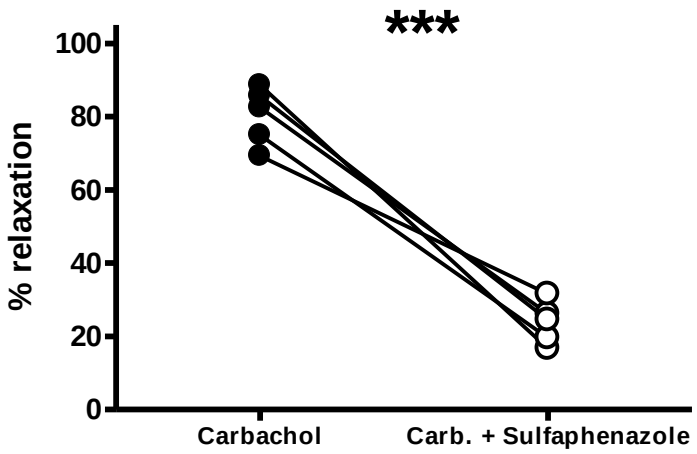

Figure 4.37. The role of CYP2C9 in the relaxation to carbachol.

The relaxation to carbachol (80.2 % ± 3.6) was inhibited by the CYP2C9 blocker sulfaphenazole (10μM) (23.7 % ± 2.6). Results show mean ± SE, n=5, *** $p < 0.001$.

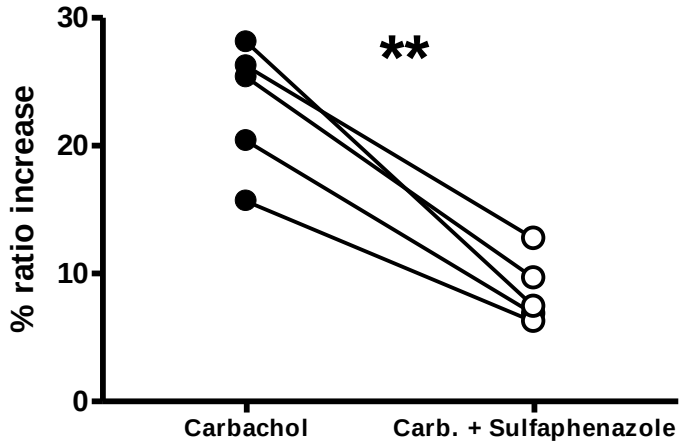

Figure 4.38. The role of CYP2C9 in the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (23.1 % ± 2.3) was inhibited by the CYP2C9 blocker sulfaphenazole (10μM) (8.6 % ± 1.2). Results show mean ± SE, n=5, ** $p < 0.01$.

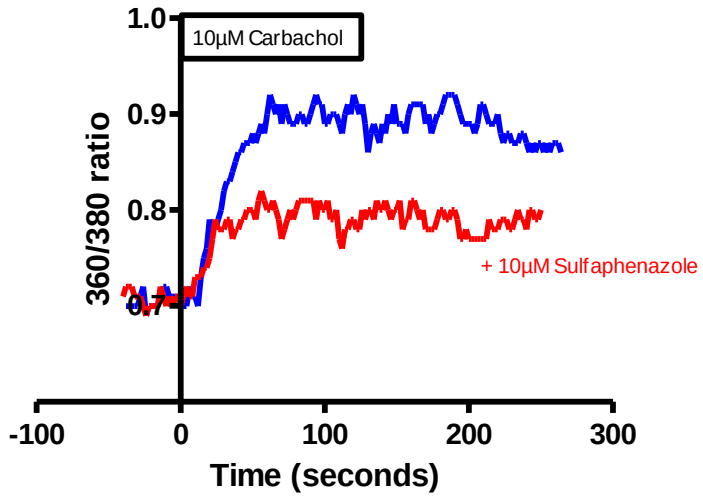

Figure 4.39. A typical trace of the 360/380nm ratio increase following carbachol application.

The CYP2C9 blocker sulfaphenazole inhibited the increase in Ca^{2+}.

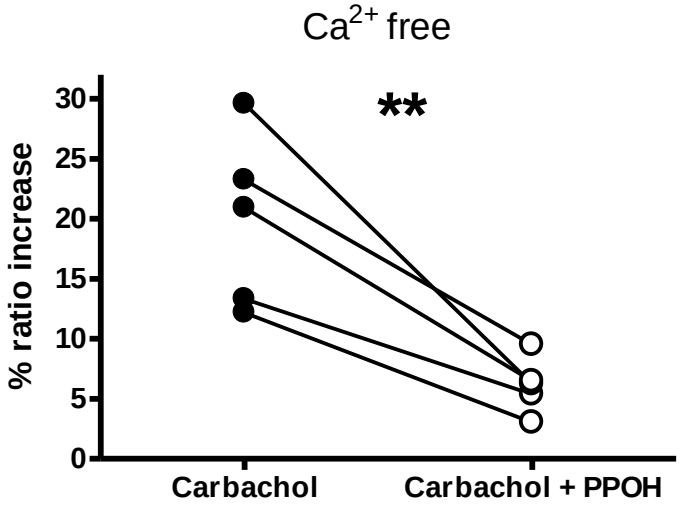

Figure 4.40. The effect of CYP450 inhibition on the 360/380nm ratio increase to carbachol in the Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (19.8 % ± 3.2) was inhibited by the CYP450 blocker PPOH (10μM) (6.1 % ± 1.0). Results show mean ± SE, n=5, ** $p < 0.01$.

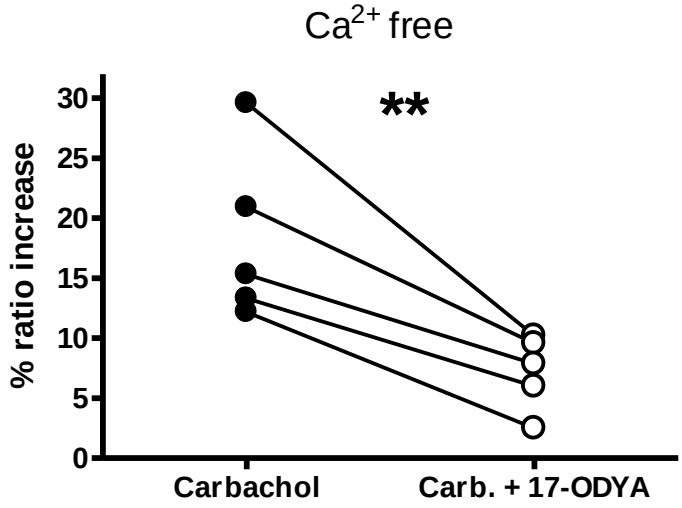

Figure 4.41. The effect of CYP450 inhibition on the 360/380nm ratio increase to carbachol in the Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (18.3 % ± 3.2) was inhibited by the CYP450 blocker 17-ODYA (50μM) (7.2 % ± 1.4). Results show mean ± SE, n=5, ** $p < 0.01$.

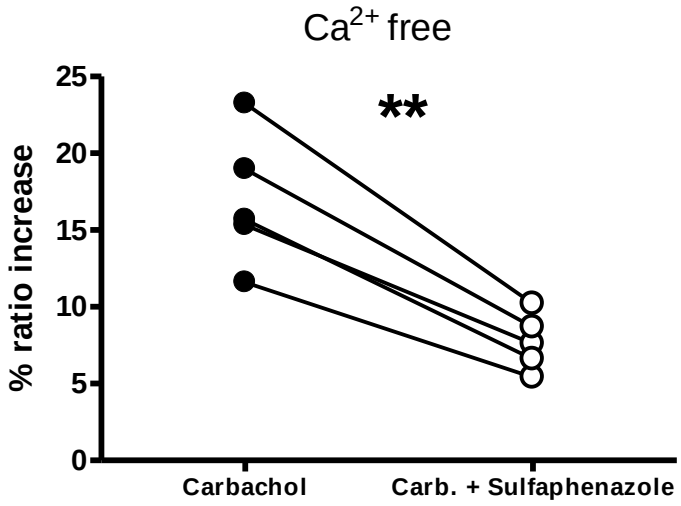

Figure 4.42. The role of CYP2C9 in the 360/380nm ratio increase in the Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (17.0 % ± 2.0) was inhibited by the CYP2C9 blocker sulfaphenazole (10µM) (7.7 % ± 0.8). Results show mean ± SE, n=5, ** $p < 0.01$.

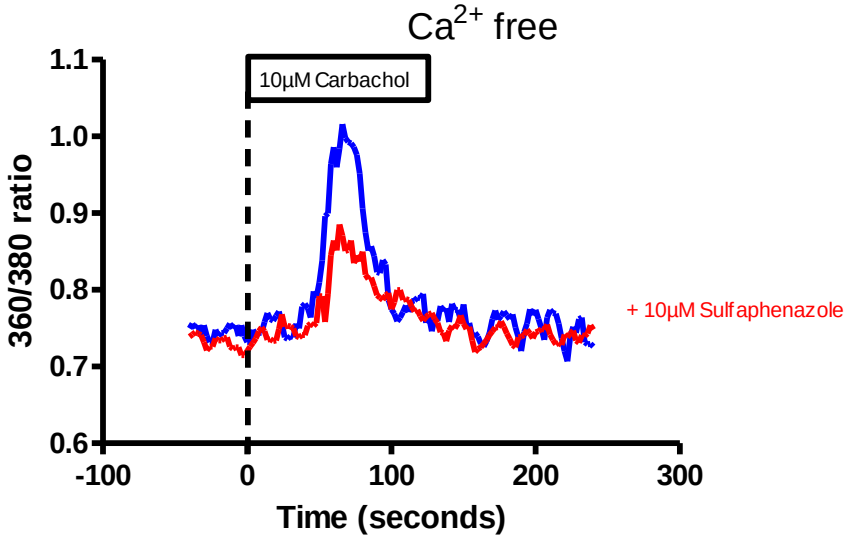

Figure 4.43. A typical trace of the increase in 360/380nm ration in response to carbachol in a Ca^{2+} free preparation.

The co-application of the CYP2C9 blocker sulfaphenazole inhibited the 360/380nm rise.

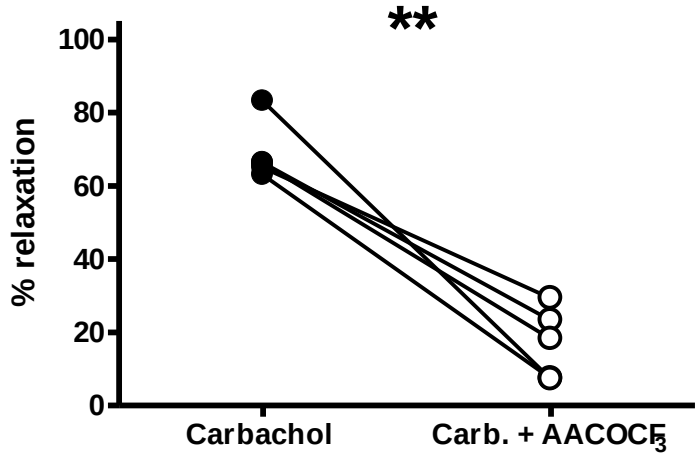

Figure 4.44. The effect of PLA_2 inhibition on relaxation to carbachol.

The % relaxation to carbachol (68.7 % ± 3.7) was inhibited by the PLA_2 blocker $AACOCF_3$ (3μM) (17.1 % ± 4.4). Results show mean ± SE, n=5, ** $p < 0.01$.

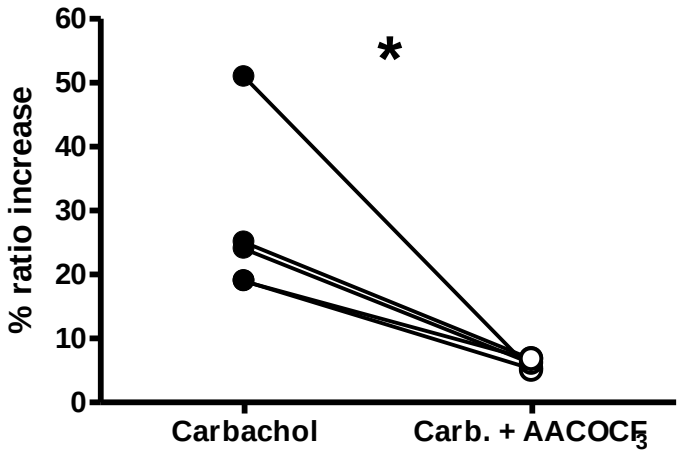

Figure 4.45. The effect of PLA_2 inhibition on the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (27.6 % ± 6.0) was inhibited by the PLA_2 blocker $AACOCF_3$ (3μM) (5.9 % ± 0.4). Results show mean ± SE, n=5 , * $p < 0.05$.

4.3.4 EETs and EDHF

EETs have been suggested to be a possible candidate for EDHF, and CYP450 enzymes are an important source of EETs. Since the experiments described in the previous section showed that CYP450 is involved in the EDHF pathway, I investigated whether this could be due to EETs rather than ROS. EEZE is a selective EETs antagonist that blocks their action by being an inactive analogue. When the preparation was pre-treated with EEZE there was no change in the EDHF response to carbachol (see Fig. 4.46 & 4.47). Figure 4.48 shows a typical trace with EEZE not affecting the Ca^{2+} rise to carbachol. This was further investigated by enhancing EET levels by blocking the enzyme which breaks down EETs using the inhibitor AUDA. It was found that enhancing EET levels with AUDA had no effect on the relaxation or endothelial cell [Ca^{2+}] rise to carbachol (see Fig. 4.49 & 4.50). Exogenous 11,12-EETs were applied to the rat cremaster arterioles and they produced a significant relaxation of over 60% (relaxation 62.9 % $\pm$ 2.3, n=5; see Fig. 4.51). However this reaction was almost completely abolished by the BK_{Ca} channel inhibitor iberiotoxin (see Fig. 4.52). As I had previously shown that iberiotoxin had no effect on the EDHF response to carbachol (Section 3.3.5), these results suggested that though exogenous 11,12-EETs can lead to relaxation via activation of BK_{Ca} channels, in this tissue the channels are not involved in the EDHF response.

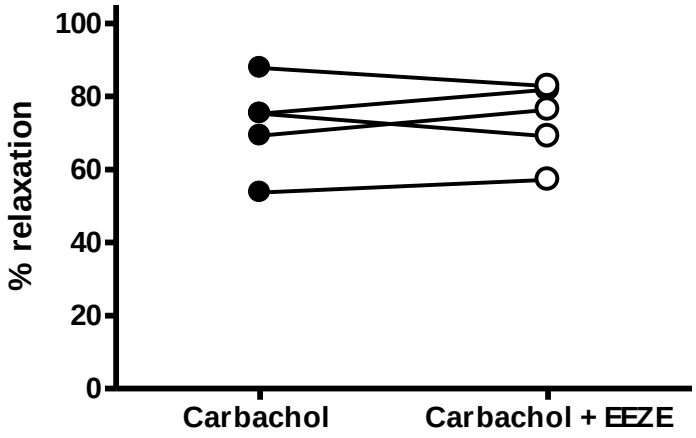

Figure 4.46. The effect of blocking EETs on the relaxation to carbachol.

The relaxation to carbachol (72.3 % ± 5.5) was not affected by the EETs blocker EEZE (10µM) (73.4 % ± 4.8). Results show mean ± SE, n=5, (n.s)

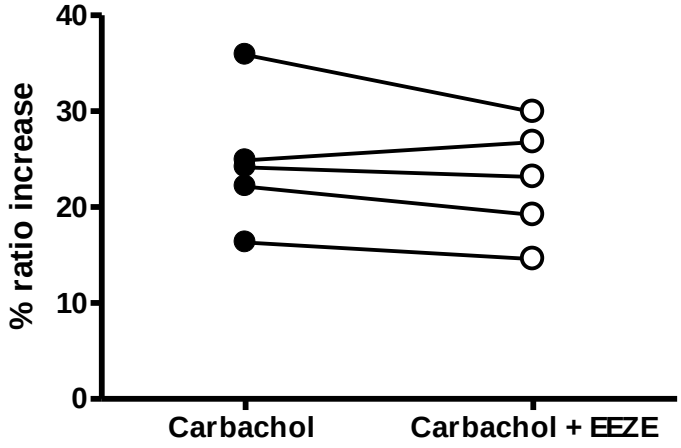

Figure 4.47. The effect of blocking EETs on the 360/380nm ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (24.7 % ± 3.2) was not significantly affected by the EETs blocker EEZE (10µM) (22.7 % ± 2.7). Results show mean ± SE, n=5, (n.s)

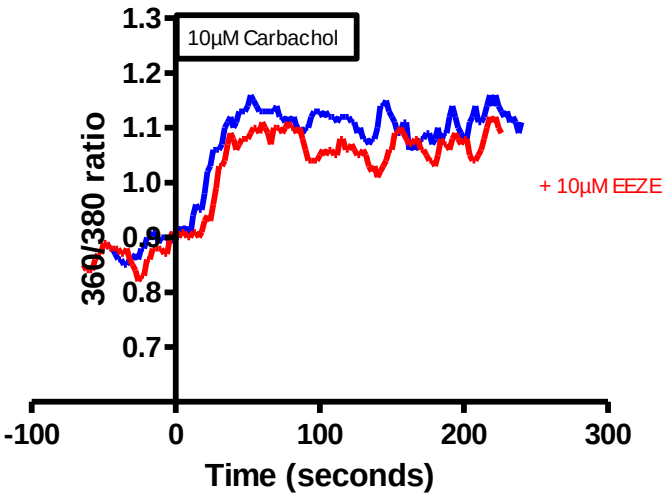

Figure 4.48. A typical trace showing the lack of effect of EEZE on the increase in the 360/380 ratio in response to carbachol application.

The EETs antagonist EEZE had no affect on the Ca^{2+} rise to carbachol.

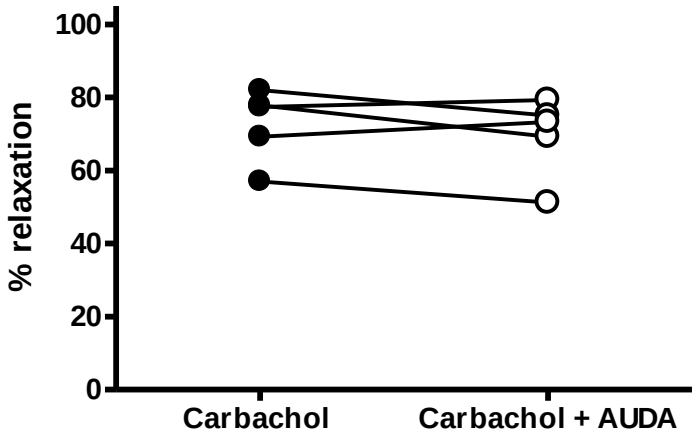

Figure 4.49. The effect of sEH inhibition, which will block the breakdown of EETs, on the relaxation to carbachol.

The relaxation to carbachol (72.7 % ± 4.4) was not affected by the sEH blocker AUDA (1μM) (69.6 % ± 4.9). Results show mean ± SE, n=5, (n.s).

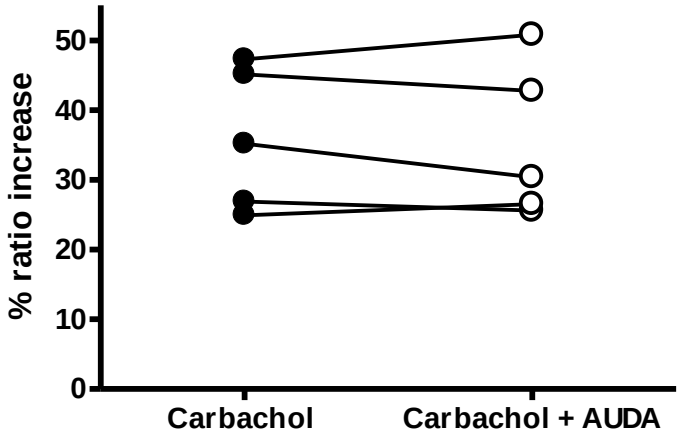

Figure 4.50. The effect of sEH inhibition on the ratio increase to carbachol.

The 360/380nm ratio increase to carbachol (35.9 % ± 4.6) was not changed by the sEH blocker AUDA (1μM) (35.2 % ± 4.0). Results show mean ± SE, n=5, (n.s).

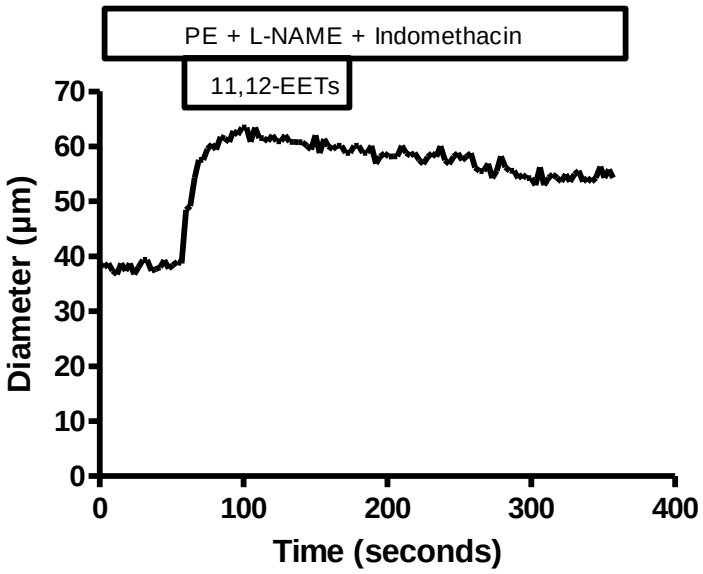

Figure 4.51. Typical trace of the relaxation to 11,12-EETs.

Trace shows the change in diameter in arterioles pre-constricted with phenylephrine in the presence of L-NAME and indomethacin showing relaxation following the addition of 11,12-EETs (1µM).

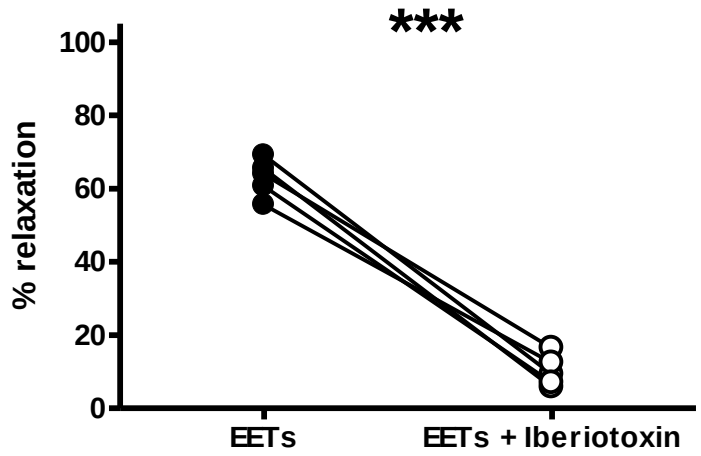

Figure 4.52. The role of BK_{Ca} channels in the relaxation to 11,12-EETs.

The relaxation to 11,12-EETs (1µM) (62.9 % $\pm$ 2.3) was significantly inhibited by the BK_{Ca} channel blocker iberiotoxin (100nM) (10.1 % $\pm$ 1.9). Results show mean $\pm$ SE, n=5, *** $p < 0.001$.

4.4 Discussion

4.4.1 The role of ROS in the EDHF response

These results demonstrated the important role of ROS in the EDHF response, with the antioxidants SOD and catalase blocking the relaxation and endothelial cell [Ca^{2+}] rise by >50%. This was confirmed by the electrophysiology study which showed the antioxidants strongly inhibited the smooth muscle hyperpolarisation to carbachol. The relaxation and [Ca^{2+}] rise to carbachol were potentiated in the presence of the catalase inhibitor Atz, further supporting the role of ROS in the response. The experiments conducted in the absence of extracellular Ca^{2+} demonstrated that the release of Ca^{2+} from intracellular stores is also partly ROS dependent, with the Ca^{2+} rise halved in the presence of SOD and catalase. That the action of the ROS was on Ca^{2+} release was supported by SOD and catalase having no effect on the relaxation to the SK_{Ca} and IK_{Ca} channel opener NS309, which suggests that the ROS are exerting their action on the endothelial cells at a step before these channels open.

The ROS may be acting to potentiate Ca^{2+} release from endothelial stores. This could be through redox modification of the IP_3 receptor which makes it more sensitive to activation by IP_3, most likely via oxidation of thiol groups on the cysteine residues of the IP_3 receptor. This means that in the presence of ROS, Ca^{2+} release from the stores would be enhanced. This increase in endothelial cell Ca^{2+} would lead to the activation of the IK_{Ca} and SK_{Ca} channels and the hyperpolarisation of the endothelial cell, and the subsequent hyperpolarisation and relaxation of the smooth muscle.

The role of ROS in potentiating rather than causing the EDHF response would explain why the antioxidants only partially block the response rather than abolishing it, but supports previous findings which have demonstrated the importance of ROS in the EDHF response. In mouse mesenteric arteries, antioxidants were shown to block the EDHF response to acetylcholine (Matoba et al., 2000). The potentiation by ROS of Ca^{2+} release from stores due to redox modification of the IP_3 receptor was suggested by Edwards and colleagues, who showed that H_2O_2 enhanced the store release of Ca^{2+}, and this was mimicked by a the use of a thiol oxidant (Edwards et al., 2008). The finding that ROS may be acting on the endothelium rather than the smooth muscle during the EDHF response has been shown in other tissues as well; for example H_2O_2 was required for the EDHF response in human gut mucosal arteries but did not itself cause smooth muscle relaxation (Hatoum et al., 2005). These results suggest that rather than being the EDHF itself, ROS potentiate the response by enhancing the release of Ca^{2+} from intracellular stores.

4.4.2 ROS species

I also attempted to determine which ROS species was involved in the EDHF response. In the Ca^{2+} free preparation, SOD alone had no effect on the response suggesting that superoxide is not the active species. On the other hand catalase alone did reduce the Ca^{2+} release from stores, suggesting that H_2O_2 is involved in the response. However, in this case it might have been predicted that SOD would have enhanced the response as this should have increased H_2O_2 production. One possible explanation for this lack of effect was that SOD levels in the tissue were already sufficiently high to ensure maximal conversion of superoxide to H_2O_2.

The use of DFO and the iron chelators CP85 and CP94 indicates that iron is important for the EDHF mediated relaxation and the rise in [Ca^{2+}] during the EDHF response. The iron chelators significantly inhibited relaxation and reduced the [Ca^{2+}] rise caused by carbachol. This suggests a role for the Fenton reaction (1) which coverts H_2O_2 to the more reactive hydroxyl radical ($^{\cdot}OH$**)** and requires iron:

(1) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$

Although DFO chelates iron, it also scavenges ROS. However, the hydroxypyridine iron chelators are more specific and have a greater affinity for iron, providing clearer evidence for involvement of the Fenton reaction (Kayyali et al., 1998). Other research has found that following generation of the hydroxyl radical there is an increase in intracellular [Ca^{2+}] (Az-ma et al., 1999). Taken together, these results imply that conversion of H_2O_2 to hydroxyl radical could be important for raising intracellular Ca^{2+} and thus causing EDHF-mediated relaxation, although work by others suggests that it is H_2O_2 rather than the hydroxyl radical that is important in raising intracellular Ca^{2+} (Volk et al., 1997). However the use of the iron chelator ophenanthroline in the Volk study may not have significantly inhibited the Fenton reaction within the cell, as it is not as cell permeant as CP94. CP94 strongly (80%) inhibits the transient rise in Ca^{2+} following carbachol application, while CP85 had no effect. CP94 is cell permeant so it can block intracellular hydroxyl radical generation by the Fenton reaction. This suggests that is the hydroxyl radical that is acting to potentiate release from the Ca^{2+} stores. This is consistent with findings by Doan who reported that H_2O_2 increased intracellular [Ca^{2+}], possibly through release from stores, although this and

other studies did not investigate whether it was H_2O_2 acting directly or through its conversion the hydroxyl radical.,

On the other hand, H_2O_2 seems the more likely signalling species as it is more stable and can act specifically to target thiol groups (Janssen-Heininger et al., 2008) whereas the hydroxyl radical is unstable with a short half life so is considered not to be well suited for being a signalling molecule (Halliwell & Gutteridge, 2007). This may mean that the removal of iron by the chelators is having another effect on the pathway, separate to inhibiting the Fenton reaction, possibly by affecting the redox state of the endothelial cells.

4.4.3 Source of ROS

The results of my experiments with inhibitors of different pathways involved in ROS generation indicates that cytochrome P450 appears to be involved. Inhibition of enzymes such as NADPH oxidase and xanthine oxidase had no effect on the EDHF response, whereas using the non-specific CYP450 inhibitors PPOH and 17-ODYA significantly blocked the response to carbachol. Sulfaphenazole was used as a selective CYP2C9 inhibitor, as this isoform has been demonstrated to be a significant source of ROS in endothelial cells (Fleming et al., 2001). In the rat cremaster arterioles it almost abolished the relaxation and endothelial [Ca^{2+}] rise to carbachol. Its role as the source of ROS was supported by the observation that sulfaphenazole mimicked the antioxidants by also blocking the release of Ca^{2+} from the intracellular stores as shown in the Ca^{2+} free preparation.

Blocking $iPLA_2$ also inhibited the responses to carbachol, supporting the idea of CYP450 being the ROS generating pathway. $iPLA_2$ releases arachidonic acid from the phospholipid membrane. Arachidonic acid can then be metabolised by CYP450, specifically CYP2C9, to produce ROS and other products including EETs. This is consistent with work in porcine coronary arteries where H_2O_2 was found to cause an EDHF response, and exogenous arachidonic acid mimicked the relaxation suggesting that it was a source of ROS (Barlow et al., 1998). Inhibition of PLA_2 in rat coronary arteries almost abolished the EDHF response to bradykinin, suggesting that the mechanism involved the PLA_2 pathway in this preparation was well (Fulton et al., 1996). CYP2C9 has been shown to be a significant source of ROS and is expressed in endothelial cells with overexpression leading to an increase in ROS production (Bolz et al., 2000; Fleming et al., 2001). In human coronary arteries, a metabolite of CYP2C9 was shown to be important in the EDHF response suggesting that it is also important physiologically in humans (Larsen et al., 2008). The results together suggest that a metabolite of $iPLA_2$ – CYP2C9 pathway is important in the EDHF response through the release of Ca^{2+} from intracellular stores.

4.4.4 EETs

As EETs are also produced following the metabolism of arachidonic acid by CYP2C9, they rather than H_2O_2 could be the active product. I therefore investigated their role in the response to carbachol using the EETs inhibitor EEZE. However, EEZE had no effect on the EDHF response to carbachol, and moreover blocking EETs breakdown with AUDA did not potentiate the response either.

On the other hand, the application of exogenous EETs did lead to a relaxation in the rat cremaster arterioles. This response was abolished by blocking the BK_{Ca} channels with iberiotoxin. However, since as I described in Chapter 3 the EDHF response in this tissue is working primarily through the IK_{Ca} and SK_{Ca} channels rather than the BK_{Ca} channel, my results suggest that while EETs can cause relaxation, they are not involved in the EDHF response in rat cremaster arterioles. There is evidence in rat mesenteric arteries of EETs being EDHF and that EEZE blocks the response (Fleming et al., 2007). However another study in mouse ductus arteries found that a CYP450 metabolite was involved in the EDHF response, but using mass spectrometry did not find an increase in EETs so they suggest that it was another product of the CYP450 pathway (Baragatti et al., 2009). These results suggest that there are tissues and species differences but that in the rat cremaster arterioles, EETs are not the active product from the CYP450 pathway.

4.4.5 Summary

These results demonstrate an important role for ROS in the EDHF response, and this primarily seems to be through potentiating the release of Ca^{2+} from intracellular stores. I conclude that the ROS therefore are acting on the endothelial cells to enhance the EDHF response, rather than being the factor which is released by the endothelium and acts on the smooth muscle. The inhibition of the response with iron chelators suggests that the hydroxyl radical could be important in the pathway, however the weight of previous evidence is behind H_2O_2 being the signalling molecule due to is properties. The $iPLA_2$ and CYP2C9 pathway appears to be the major source of the

ROS, and EETs do not appear to be important in the EDHF response in the rat cremaster arterioles.

Chapter 5: The effect of H_2O_2 in rat cremaster arterioles.

5.1 Introduction

5.1.1 Characterising the response to H_2O_2

In this chapter, I describe experiments in which the effect of exogenous H_2O_2 on vascular diameter and endothelial [Ca^{2+}] was measured in rat cremaster arterioles, and the mechanisms behind these responses were examined in a manner similar that used to characterise the carbachol response – in other words by employing various pharmacological blockers. H_2O_2 has been suggested to be an EDHF, and much of this work has been based on the addition of exogenous H_2O_2 (Matoba et al., 2000). In previous chapters it has been shown that ROS play an important role in the EDHF response, and they appear to be acting in part through the release of Ca^{2+} from intracellular stores. The use of exogenous H_2O_2 allows this response to be compared to that of carbachol, and the determination of whether these share the same properties and susceptibilities to different blockers.

H_2O_2 has been shown to be able to directly cause relaxation in a number of different vascular beds. It has been shown to act directly on the BK_{Ca} channels on VSM to cause K^+ efflux and hyperpolarisation which leads to relaxation (Sobey et al., 1998). It also may be able to directly open other channels, such as the IK_{Ca} and SKCa channels and the redox sensitive TRPM2 channel (Hequet et al., 2008; Winterbourn et al., 2008). PKG has been shown to be redox sensitive and exogenous H_2O_2 can directly activate the kinase leading to relaxation in the VSM cell using a similar mechanism to NO (Burgoyne et al., 2007). These pathways by which H_2O_2 could be causing relaxation were studied using different inhibitors.

5.1.2 H_2O_2 and the carbachol response

In previous chapters I have shown that ROS are involved in the EDHF response to carbachol with antioxidants inhibiting the relaxation and rise in endothelial [Ca^{2+}]. To investigate the role of H_2O_2 during the response to carbachol, exogenous H_2O_2 was added. The concentrations of H_2O_2 used in these experiments had no effect on relaxation or endothelial [Ca^{2+}] levels when applied on its own (see Fig. 5.1). This ensured that only any effect on the carbachol response would be seen.

Sulfaphenazole, the CYP2C9 blocker, was shown to inhibit the EDHF response to carbachol. This could be because CYP2C9 is the important source of ROS during the response. To investigate this H_2O_2 was added in the presence of sulfaphenazole to see if it could rescue the EDHF response to carbachol by providing an alternate source of ROS.

5.2 Methods

The effect of H_2O_2 on rat cremaster arterioles was characterised using intravital microscopy to measure changes in vessel diameter and endothelial cell Ca^{2+} levels. The preparation used is described in the General Methods chapter.

5.2.1 Protocol

The arterioles were pre-constricted with 30µM phenylephrine with 300µM L-NAME and 3µM indomethacin present. This was to ensure that the only the effects of H_2O_2

which were independent of NO and PGI_2 would be observed. H_2O_2 was applied for 2 minutes to elicit a response and there was a 15 minute wash off period between experiments. Different inhibitors were then used to characterise the mechanism behind the H_2O_2 response in the arterioles. The preparation was pre-treated with the drugs for 2 minutes other than 5 minutes for apamin and TRAM-34, 10 minutes for $AACOCF_3$ and 1 hour perfusion with the TRPM2 antibody.

5.2.2 Analysis

The responses to H_2O_2 (% relaxation and % 360/380nm ratio increase) are paired with the response with the inhibitors present in the same arteriole to allow for direct comparison. These paired results are displayed in the graphs and were analysed with a two-tailed paired t-test. The difference between the compared results was considered significant if the p value was less than 0.05, represented by a * on the graph (** $p<0.01$, *** $p<0.001$).

5.3 Results

5.3.1 H_2O_2 mediated relaxation

Concentration-response experiments with application of exogenous H_2O_2 demonstrated that 100µM was required to give a consistent relaxation comparable to 10µM carbachol (relaxation 73.7 % $\pm$ 1.4, n=16; see Fig. 5.1 & 5.2). 100µM H_2O_2 also caused a significant increase in endothelial cell Ca^{2+} (ratio increase 23.0 % $\pm$ 1.2, n=16; see Fig. 5.3). This suggests that although quite high concentrations are required, H_2O_2 can directly cause relaxation in rat cremaster arterioles.

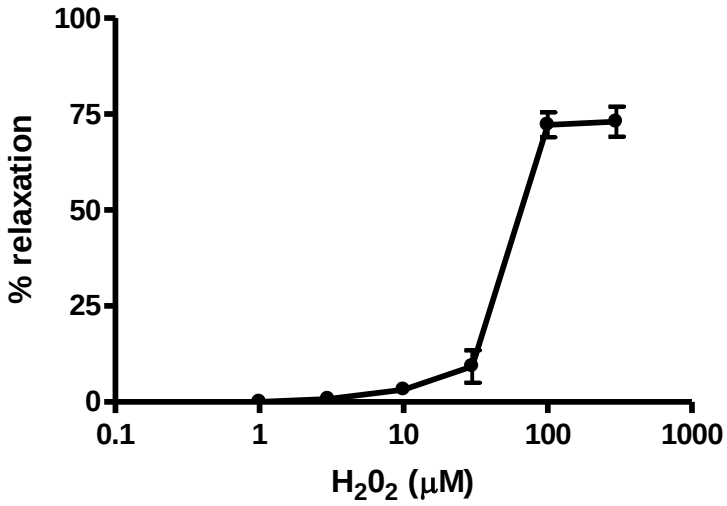

Figure 5.1. Concentration-dependent relaxation of rat cremaster arterioles to exogenous H_2O_2.

Graph showing the % relaxation to the addition of exogenous H_2O_2 (1-300μM). Results are mean ± standard error of mean, n=5.

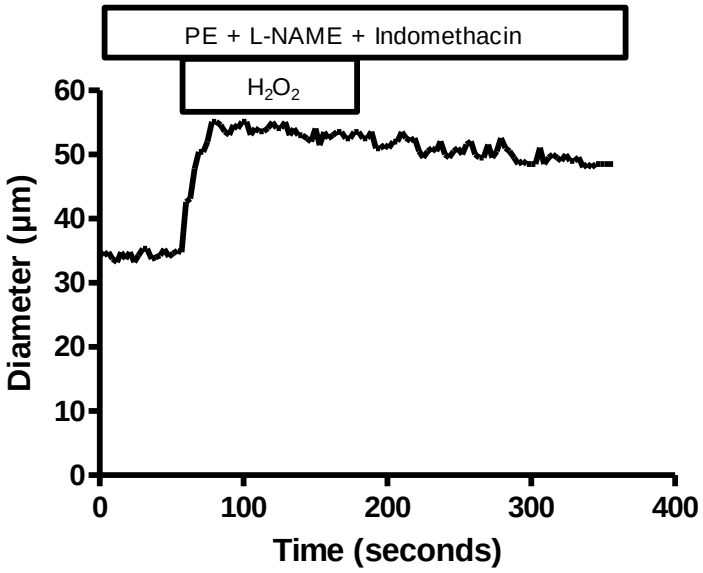

Figure 5.2. Typical trace showing vasodilatation of PE-preconstricted rat cremaster arteriole to 100 µM H_2O_2.

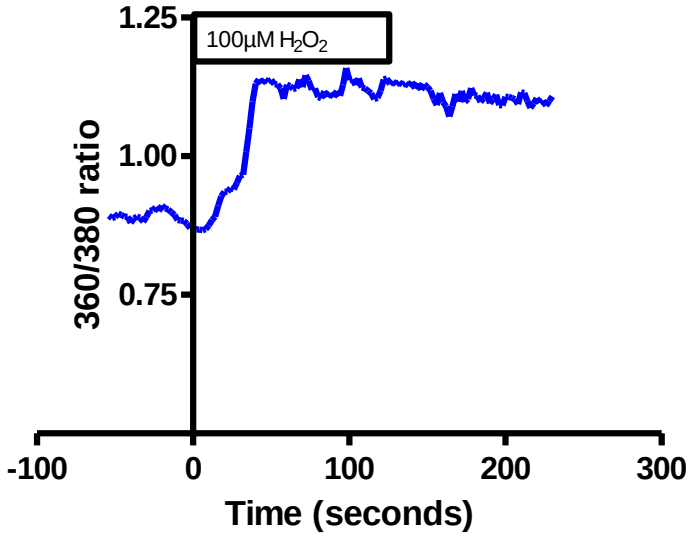

Figure 5.3. Typical trace of the increase in 360/380nm ratio to H_2O_2

5.3.2 H_2O_2 and EDHF response

To investigate whether exogenous H_2O_2 is working through the same mechanisms as the EDHF response various inhibitors were co-applied. The SK_{Ca} and IK_{Ca} channel blockers apamin and TRAM-34 have previously been shown to almost abolish the EDHF response to carbachol. With H_2O_2 application, thse drugs caused a partial inhibition of the relaxation and rise in $[Ca^{2+}]_i$, suggesting in part that H_2O_2 is causing relaxation through the opening of IK_{Ca} and SK_{Ca} channels (see Fig. 5.4 & 5.5). Addition of the BK_{Ca} channel blocker iberiotoxin was found to strongly inhibit the relaxation to H_2O_2, whereas it has no effect on the carbachol induced relaxation (see Fig. 5.6 & 5.7). Similarly, the PKG inhibitor Rp-8-Bromo-cGMP which had no effect on the carbachol response, did greatly inhibit the relaxation in response to H_2O_2 (see Fig. 5.8 & 5.9). PLA_2, was suggested to be part of the ROS producing pathway (see section 4.3.3). Its inhibition had no affect on the response to H_20_2 (see Fig. 5.10 & 5.11). The TRPM2 blocker ACA and the TRPM2 antibody which blocks the cation channel also significantly inhibited the relaxation and also the $[Ca^{2+}]$ rise in response to H_2O_2 (see Fig. 5.12, 5.13, 5.14 & 5.15). The iron chelator CP94 had no affect on the response to H_2O_2 suggesting that it H_2O_2 is the reactive species rather than hydroxyl radicals (see Fig. 5.16 & 5.17). These observations suggest that exogenous H_2O_2 causes relaxation by a number of mechanisms.

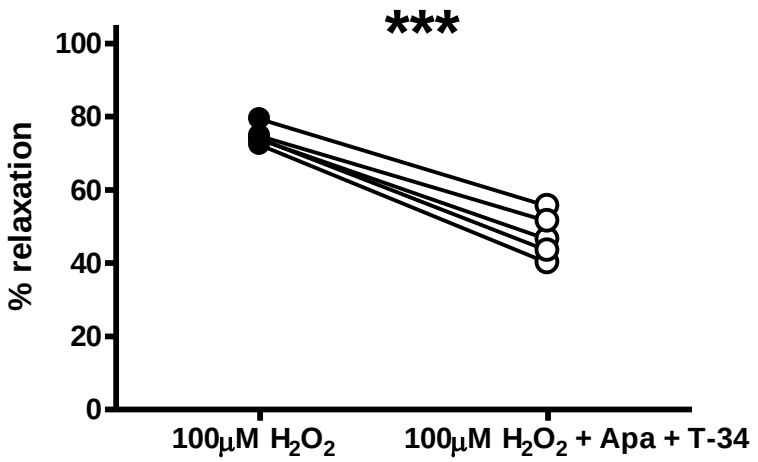

Figure 5.4. The role of the SK_{Ca} and IK_{Ca} channels in the relaxation to H_2O_2.

The relaxation to H_2O_2 (74.8 % $\pm$ 1.2) was inhibited by the SK_{Ca} and IK_{Ca} blockers apamin (500nM) and TRAM-34 (10μM) (47.3 % $\pm$ 2.8). Results show mean $\pm$ SE, n=5, *** $p < 0.001$.

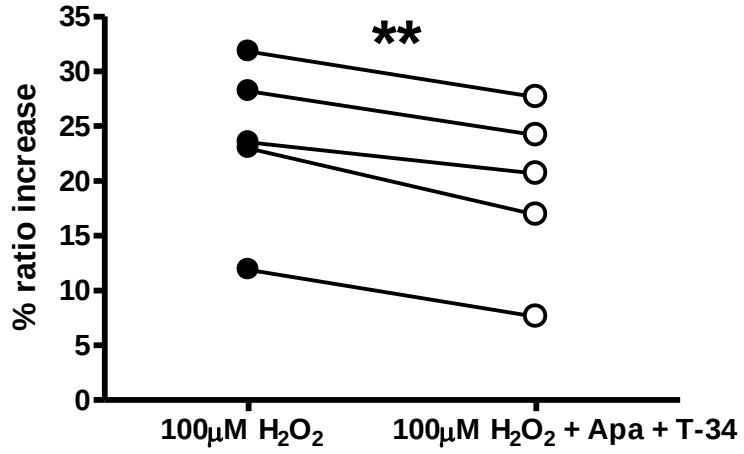

Figure 5.5. The role of SK_{Ca} and IK_{Ca} channels in the ratio increase to H_2O_2.

The 360/380nm ratio increase to H_2O_2 (23.7 % $\pm$ 3.4) was partially inhibited by the SK_{Ca} and IK_{Ca} blockers apamin (500nM) and TRAM-34 (10μM) (19.4 % $\pm$ 3.4). Results show mean $\pm$ SE, n=5, ** $p < 0.01$.

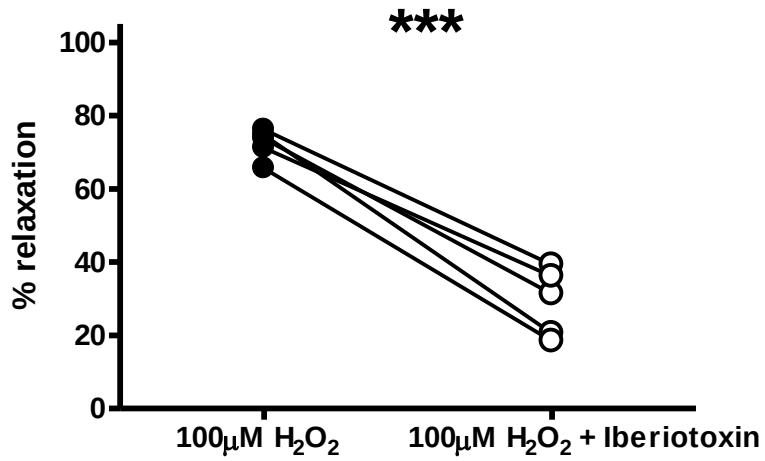

Figure 5.6. The role of BK_{Ca} channels in the relaxation to H_2O_2.

The relaxation to H_2O_2 (72.3 % ± 1.9) was attenuated by the BK_{Ca} channel blocker iberiotoxin (100nM) (29.1 % ± 4.1). Results show mean ± SE, n=5, *** $p < 0.001$.

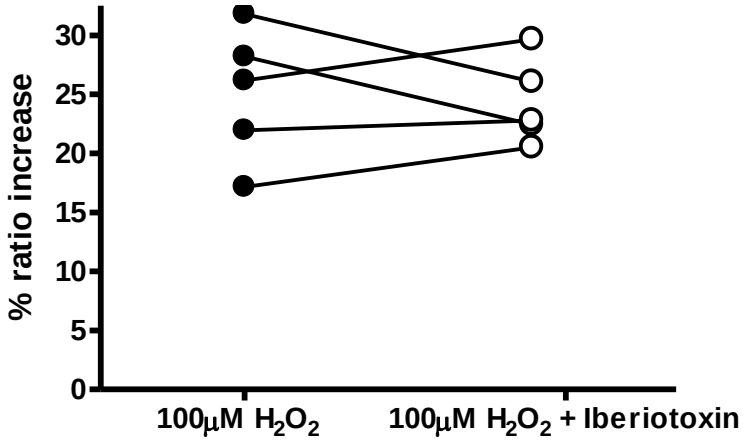

Figure 5.7. The role of BK_{Ca} channels in the ratio increase to H_2O_2.

The 360/380nm ratio increase to H_2O_2 (25.1 % ± 2.5) was not significantly affected by the BK_{Ca} channel blocker iberiotoxin (100nM) (24.3 % ± 1.6). Results show mean ± SE, n=5, (n.s).

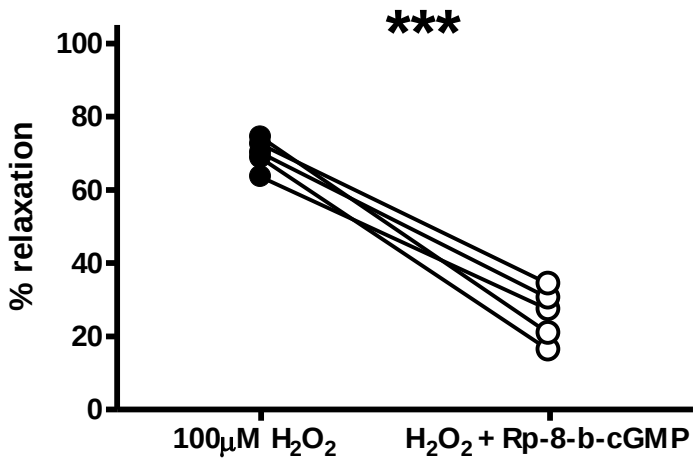

Figure 5.8. The role of PKG in the H_2O_2 mediated relaxation.

The relaxation to H_2O_2 (69.8 % ± 1.8) was significantly inhibited by the PKG inhibitor Rp-8-bromo-cGMP (100μM) (25.8 % ± 3.3). Results show mean ± SE, n=5, *** $p < 0.001$.

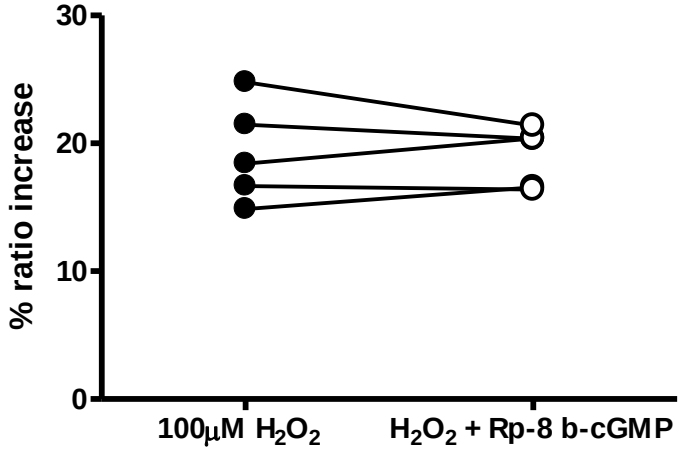

Figure 5.9. The role of PKG in the ratio increase to H_2O_2.

The 360/380nm ratio increase to H_2O_2 (19.2 % ± 1.8) was not affected by the PKG blocker Rp-8-bromo-cGMP (100μM) (19.0 % ± 1.1). Results show mean ± SE, n=5, (n.s).

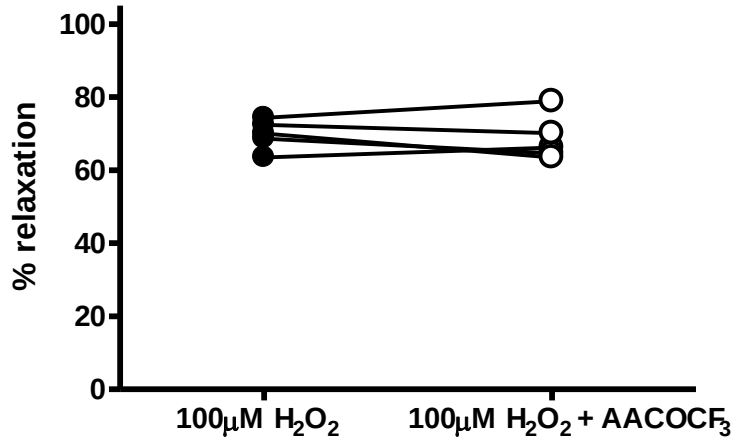

Figure 5.10. The role of PLA_2 in the relaxation to H_2O_2.

The relaxation to H_2O_2 (69.8 % $\pm$ 1.8) was not affected by the PLA_2 inhibitor $AACOCF_3$ (3μM) (68.7 % $\pm$ 2.9). Results show mean $\pm$ SE, n=5, (n.s).

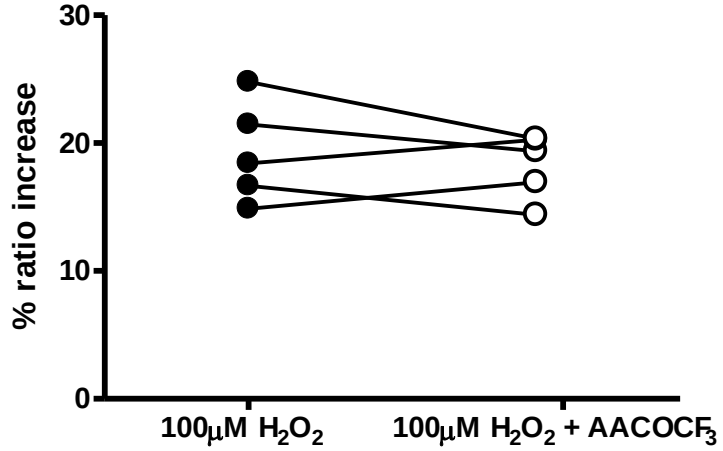

Figure 5.11. The role of PLA_2 in the ratio increase to H_2O_2.

The 360/380nm ratio increase to H_2O_2 (19.2 % $\pm$ 1.8) was not changed by the PLA_2 blocker $AACOCF_3$ (3μM) (18.3 % $\pm$ 1.1). Results show mean $\pm$ SE, n= 5, (n.s).

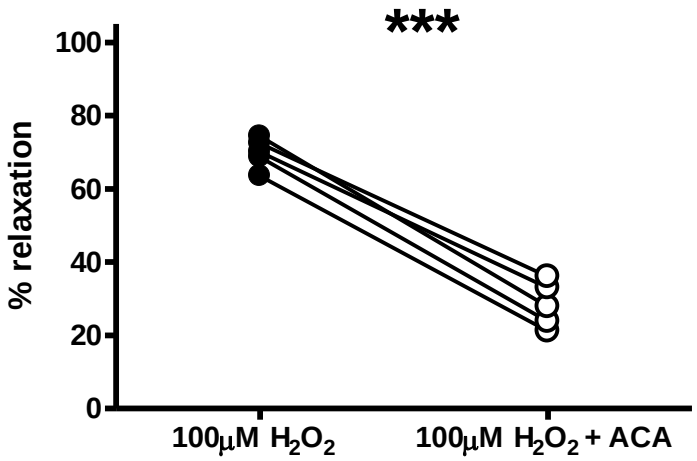

Figure 5.12. The role of TRPM2 channels in the relaxation to H_2O_2.

The H_2O_2 relaxation (69.8 % ± 1.8) was significantly inhibited by the TRPM2 blocker ACA (20μM) (28.3 % ± 2.8). Results show mean ± SE, n=5, *** $p < 0.001$.

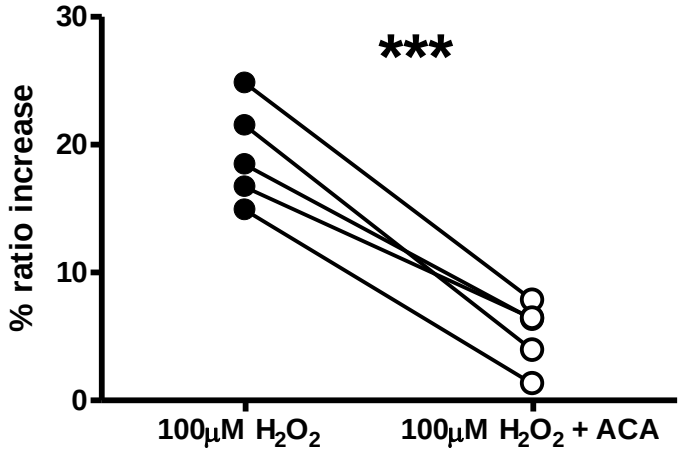

Figure 5.13. The role of TRPM2 channels in the ratio increase to H_2O_2.

The 360/380nm ratio increase to H_2O_2 (19.2 % ± 1.8) was attenuated by the TRPM2 blocker ACA (20μM) (5.1 % ± 1.2). Results show mean ± SE, n=5, *** $p < 0.001$.

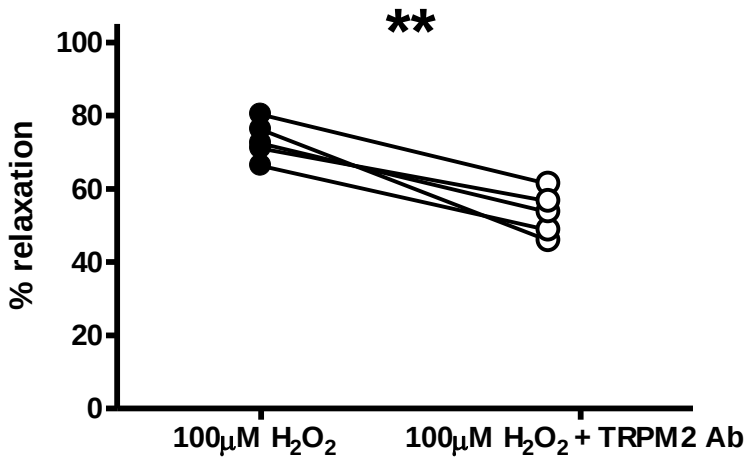

Figure 5.14. The role of the TRPM2 channel in the relaxation to H_2O_2.

The relaxation to H_2O_2 (73.2 % $\pm$ 2.4) was partially blocked by the TRPM2 antibody (5μg/ml) which blocks the TRPM2 channel (53.2 % $\pm$ 2.8). Results show mean $\pm$ SE, n=5, ** $p < 0.01$.

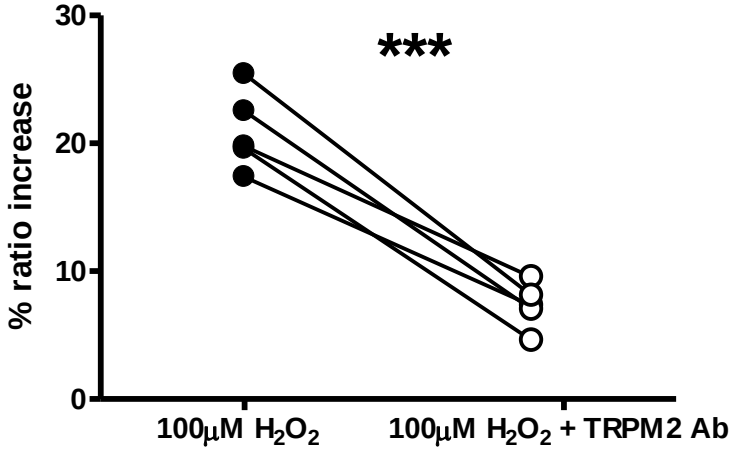

Figure 5.15. The role of the TRPM2 channel in the ratio increase to H_2O_2.

The 360/380nm ratio increase to H_2O_2 (20.9 % $\pm$ 1.4) was inhibited by the TRPM2 antibody (5μg/ml) which blocks the TRPM2 channel (7.3 % $\pm$ 0.8). Results show mean $\pm$ SE, n=5, *** $p < 0.001$.

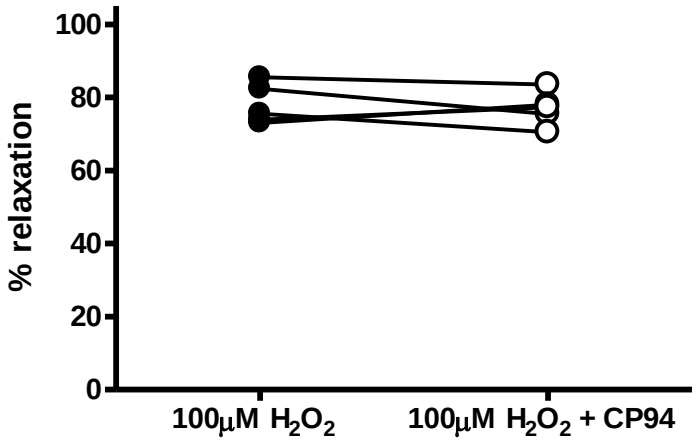

Figure 5.16. The effect of iron chelation on the relaxation to H_2O_2.

The relaxation to H_2O_2 (78.1 % ± 2.5) was not significantly affected by the iron chelator CP94 (100μM) (77.0 % ± 2.1). Results show mean ± SE, n=5, (n.s).

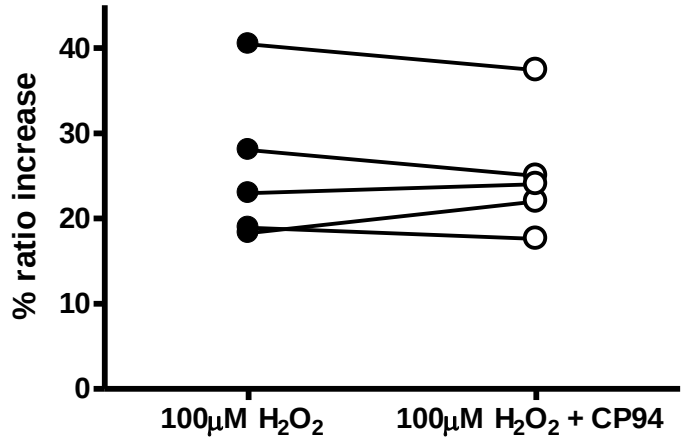

Figure 5.17. The effect of iron chelation on the ratio increase to H_2O_2.

The 360/380nm ratio increase to H_2O_2 (25.7 % ± 4.1) was not significantly changed by the iron chelator CP94 (100μM) (25.2 % ± 3.3). Results show mean ± SE, n=5, (n.s).

5.3.3 H_2O_2 and carbachol response

As shown in section 4.3.3, inhibition of CYP2C9 by sulfaphenazole almost abolished the EDHF response. I have also shown that ROS scavenging inhibited the response to carbachol, while increasing ROS levels by reducing the breakdown of H_2O_2 using a catalase inhibitor enhanced it. This suggests that a role for ROS in the EDHF response and that CYP2C9 inhibition blocks their production. To investigate whether the response could be recovered by the addition of ROS, a lower concentration of H_2O_2, which had little effect on its own, was applied with carbachol in the presence of sulfaphenazole. It was found that the co-application of 10µM H_2O_2 rescued the response to carbachol (see Fig. 5.18 & 5.19). 10µM H_2O_2 also rescued the carbachol response when PLA_2 was inhibited (see Fig. 5.20 & 5.21). To support this finding, in a Ca^{2+} free preparation, 1µM H_2O_2, which has no effect on its own, when applied with carbachol, enhanced the Ca^{2+} release from stores (see Fig. 5.22).

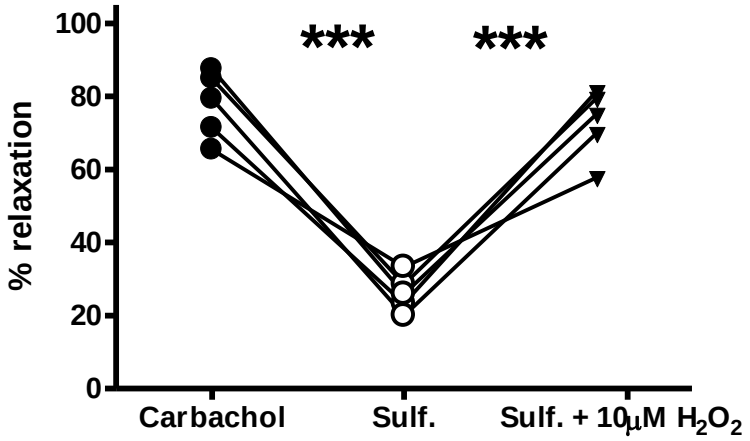

Figure 5.18. Exogenous H_2O_2 rescued the relaxation to carbachol following inhibition by sulfaphenazole.

The relaxation to carbachol (77.7 % ± 4.1) was inhibited by the CYP2C9 blocker sulfaphenazole (10μM) (26.2 % ± 2.3). This relaxation recovered when pre-treated with 10μM H_2O_2 (72.6 % ± 4.2). Results show mean ± SE, n=5, $p < 0.01$, *** $p < 0.001$.

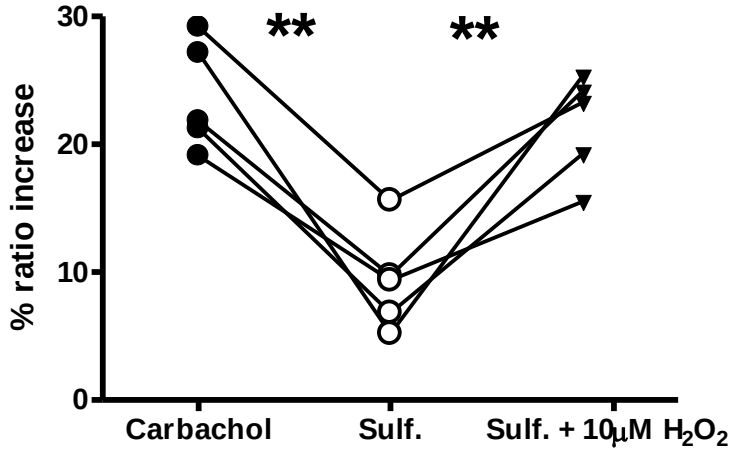

Figure 5.19. Exogenous H_2O_2 rescued the ratio increase to carbachol following inhibition by sulfaphenazole.

The 360/380nm ratio increase to carbachol (23.7 % ± 1.9) was attenuated by the CYP2C9 blocker sulfaphenazole (10μM) (9.3% ± 1.8). This inhibition by sulfaphenazole could be recovered by pre-treatment with 10μM H_2O_2 (21.5 % ± 1.8). Results show mean ± SE, n=5, ** $p < 0.01$.

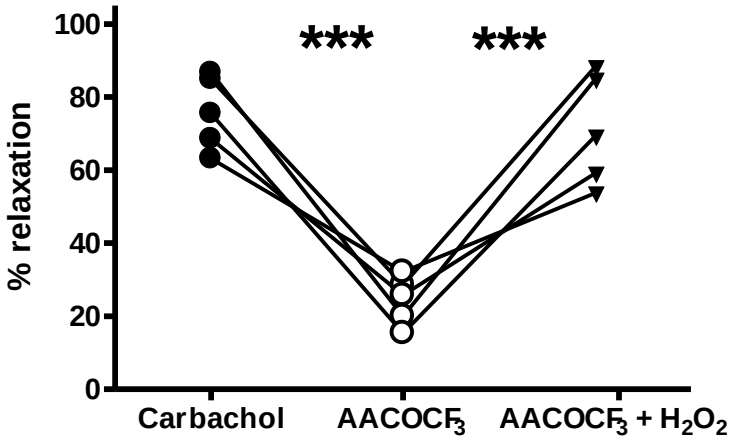

Figure 5.20. The relaxation to carbachol when inhibited by $AACOCF_3$, was rescued by H_2O_2.

The relaxation to carbachol (75.7 % ± 4.5) was inhibited by the PLA_2 blocker $AACOCF_3$ (3μM) (24.4 % ± 3.0). Pre-treatment with H_2O_2 (10μM) could recover this response (71.0 % ± 6.8). Results show mean ± SE, n=5, *** $p < 0.001$.

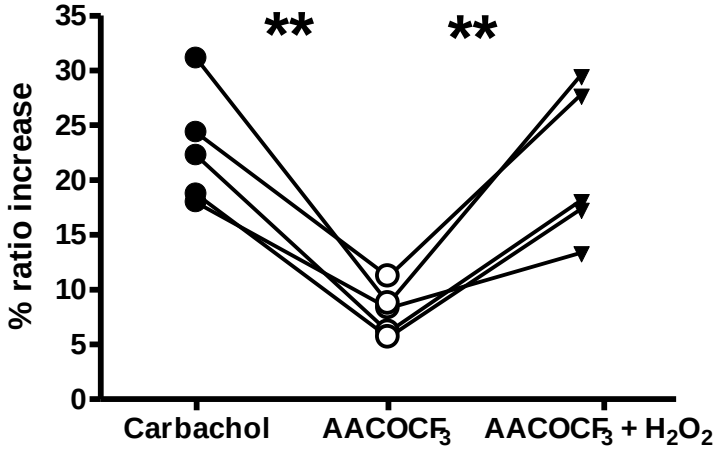

Figure 5.21. Exogenous H_2O_2 rescued the response to carbachol following PLA_2 inhibition.

The 360/380nm ratio increase to carbachol (22.8 % ± 2.4) was blocked by the PLA_2 inhibitor $AACOCF_3$ (3μM) (8.0 % ± 1.0). 10μM H_2O_2 pre-treatment recovered the response (21.2 % ± 3.1). Results show mean ± SE, n=5, ** $p < 0.01$.

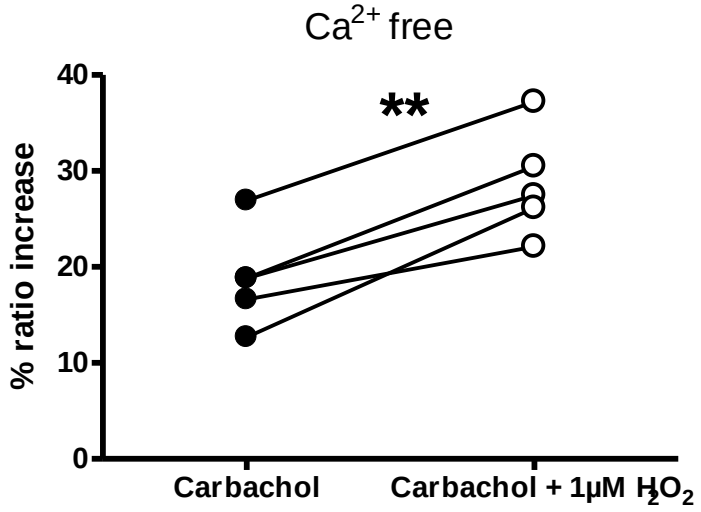

Figure 5.22. The effect of exogenous H_2O_2 on the ratio increase to carbachol in the Ca^{2+} free preparation.

The 360/380nm ratio increase to carbachol (18.8 % $\pm$ 2.3) was enhanced by pre-treatment with H_2O_2 (1µM) (28.7 % $\pm$ 2.5). Results show mean $\pm$ SE, n=5, ** $p < 0.01$.

5.4 Discussion

5.4.1 H_2O_2 response in rat cremaster

The dose response curve to H_2O_2 demonstrates that 100µM exogenous H_2O_2 was required to give a consistent good relaxation in rat cremaster arterioles. This is a high concentration of H_2O_2 to add, but is entirely in keeping with the concentrations used in other studies where 100-300µM H_2O_2 was needed to elicit substantial responses (Matoba et al., 2000; Hecquet et al 2008). This has consequences for the relevance of the conclusions that can be drawn from the results as these concentrations are probably much higher than physiological levels. However, microdomains may be able to exist where there are locally high concentrations of H_2O_2 and catalase action may mean that the H_2O_2 concentration when measured in the tissue as a whole is much lower than it is in certain compartments (Forman et al., 2004). The iron chelator CP94 had no effect on the response to H_2O_2 suggesting that it is the H_2O_2 itself that is active, rather than its metabolite the hydroxyl radical.

The relaxation to H_2O_2 was found to be partially blocked by TRAM-34 and apamin, suggesting that H_2O_2 is directly activating IK_{Ca} and SK_{Ca} channels, presumably leading to endothelial cell and VSM hyperpolarisation. Iberiotoxin inhibited the relaxation more strongly, implying that H_2O_2 is partly operating through direct opening of the BK_{Ca} channels on the smooth muscle leading to hyperpolarisation and relaxation. This is supported by others who have shown that H_2O_2 can directly open the BK_{Ca} channel through redox modification with the response blocked by catalase and iberiotoxin in rat cerebral arteries (Sobey et al., 1998). However other studies

have shown that H_2O_2 can actually reduce the open probability of the BK_{Ca} channels where a reducing agent instead enhanced opening (DiChiara et al., 1997). In human coronary arterioles, shear stress mediated relaxation was found to be caused by H_2O_2 through BK_{Ca} channel activation (Liu et al., 2011). This suggests there could be some tissue differences, and possibly variation depending on the concentration of H_2O_2 used.

The activation of PKG by cGMP is involved in the NO and PGI_2 mediated relaxation pathways. It has been shown previously that concentrations of 100μM H_2O_2 can directly activate PKG as it acts as a redox sensor. PKG is modified by H_2O_2 causing the formation of a disulfide bridge which directly activates the kinase allowing it to phosphorylate its downstream targets leading to relaxation (Burgoyne et al., 2007). In the rat cremaster arteriole, the PKG inhibitor Rp-8-bromo-cGMP strongly blocked the relaxation to H_2O_2 suggesting it is working through direct activation of PKG in the VSM by redox modification.

TRPM2 is a redox sensitive member of the TRP family of cation channels and it has been shown to be expressed in endothelial cells (Kwan et al., 2007). ACA is a non-specific TRPM2 channel inhibitor that was found to significantly block the relaxation and endothelial $[Ca^{2+}]_i$ rise to H_2O_2. To further investigate this the more specific TRPM2 antibody was used to block the channel. This also blocked the response to H_2O_2, suggesting that it opens the channel directly to increase endothelial cell $[Ca^{2+}]$. It cdo this via redox modification of TRPM2 or possibly by increasing ADP ribose formation which activates the channel. H_2O_2 has been shown in endothelial cells to

increase the open probability of the channel which was blocked by use of the antibody (Hecquet 2008).

5.4.2 H_2O_2 and carbachol

It has previously been shown that the EDHF response to carbachol is inhibited if ROS are blocked by antioxidants, or by blocking the pathways that generate them. Sulfaphenazole blocks CYP2C9 and $AACOCF_3$ $blocks_2$ $iPLA_2$, both of which appear to be involved in ROS production in the rat cremaster arterioles. I found that 10µM H_2O_2 application had no effect on the diameter or endothelial [Ca^{2+}]. However, when the arteries were pre-treated with 10µM H_2O_2, in the presence of sulfaphenazole or $AACOCF_3$, the EDHF response to carbachol was restored. This supports the finding that CYP2C9 and $iPLA_2$ are part of the ROS production pathway as H_2O_2 replaces the lost ROS and thereby rescues the response to carbachol. Further evidence of this was that 1µM H_2O_2 enhanced the store release of Ca^{2+} in response to carbachol, as shown by its effect in the Ca^{2+} free preparation. This is supported by the findings by Edwards which showed that H_2O_2 potentiated the release of Ca^{2+} from internal stores (Edwards 2008).

5.4.3 Summary

Exogenous H_2O_2 at high concentrations could directly cause relaxation and an increase in endothelial [Ca^{2+}] in rat cremaster arterioles. The H_2O_2 worked by a number of different mechanisms including opening of K_{Ca} channels, TRPM2 and activation of PKG. The EDHF mediated relaxation to carbachol, when inhibited by

blockers of CYP2C9 or $iPLA_2$, could be restored by low concentrations of H_2O_2 which themselves had no effects, supporting the idea that low concentrations of ROS, which may be more physiologically relevant than the high concentrations which I and others have shown have direct effects on VSM, can potentiate the EDHF response by acting on the endothelium.

Chapter 6: The effect of IL-1β on the EDHF response.

6.1 Introduction

In previous chapters I have shown that the EDHF response to carbachol is ROS dependent. This response could be potentiated by the addition of exogenous H_2O_2, or by increasing [ROS] by blocking its breakdown by catalase. To investigate this further, the cytokine interleukin-1β (IL-1β) was used to increase ROS production. IL-1β is a pro-inflammatory cytokine which is also involved in the immune response. It is produced by a number of cells including macrophages, fibroblasts and endothelial cells (Limatola et al., 1997). IL-1β can bind to the IL-1 cytokine receptor to activate a range of signalling pathways. It has been shown that IL-1β can increase ROS production (Woodfin et al., 2011). It does this by increasing the activation of NADPH oxidase, possibly by enhancing expression of the Nox-2 subunit (Bedard et al., 2005). This was demonstrated by NADPH oxidase inhibition blocking an increase in ROS levels in cells treated with IL-1β (Yasuhara et al., 2005). Part of the action of IL-1β may be through the stimulation of PKC, possibly through increasing PKC autophosphorylation which would lead to enhanced NADPH oxidase assembly (Limatola et al., 1997). PKC is required for p47phox phosphorylation which is needed for the translocation of the regulatory subunits within a cell to cause NADPH oxidase activation. Previous studies have shown that pre-treatment of endothelial cells with IL-1β increases ROS production within 10 minutes and could potentiate ROS dependent responses such as increasing cell permeability following bradykinin stimulation (Woodfin et al., 2011). As the EDHF response to carbachol has been shown to be ROS dependent, the tissue was pre-treated with IL-1β to see if this could potentiate the relaxation and endothelial cell [Ca^{2+}] increase. The mechanism behind IL-1β's effects was also investigated using different blockers of NADPH oxidase and its activation.

6.2 Methods

The role that IL-1β has on the EDHF response to carbachol was investigated using the intravital microscopy preparation as previously described in the general methods. This allowed the effect on relaxation and endothelial Ca^{2+} to be observed.

6.2.1 Protocol

The arterioles were pre-constricted with 30μM phenylephrine and the experiments were done in the presence of 300μM L-NAME and 3μM indomethacin. The effect of IL-1β (30pM) was measured by pre-treatment for 15 minutes with the cytokine before the addition of carbachol, this concentration was based on previous studies carried out in the Fraser Lab. The mechanism behind IL-1β was investigated by the addition of different inhibitors and their effects on the EDHF response were measured. SOD, catalase and apocynin were added 2 minutes before carbachol whereas Ro 31-8220 was pre-treated for 10 minutes. The Ca^{2+} free preparation was carried out as previously described in chapter 3 methods.

6.2.2 Analysis

The results of the EDHF response to carbachol with and without IL-1β were paired. The means $\pm$ standard error were calculated and the paired data is displayed in graphs showing the effect of IL-1β on the response to carbachol and then the effect of the different inhibitors. A two-tailed paired t-test was performed to test the difference in

the results. The difference between the compared results was considered significant if the p value was less than 0.05, represented by a * on the graph (** $p<0.01$, *** $p<0.001$).

6.3 Results

6.3.1 IL-1β and EDHF response

IL-1β has been shown to increase NADPH activation which leads to an increase in the production of ROS levels. In this preparation, NADPH oxidase does not appear to be a major source of ROS during the response to carbachol under basal conditions. However, conditions designed to increase ROS levels have enhanced the response. The preparation was pre-treated with IL-1β for 15 minutes. The EDHF response to carbachol was found to be increased with a larger relaxation and increased endothelial cell Ca^{2+} rise (see Fig. 6.1 & 6.2). A typical trace shows this enhanced increase in Ca^{2+} in response to carbachol when pre-treated with IL-1β (see Fig. 6.3). This was supported by repeating the experiment in the Ca^{2+} free preparation which showed an increase in the release of Ca^{2+} from endothelial stores (see Fig. 6.4).

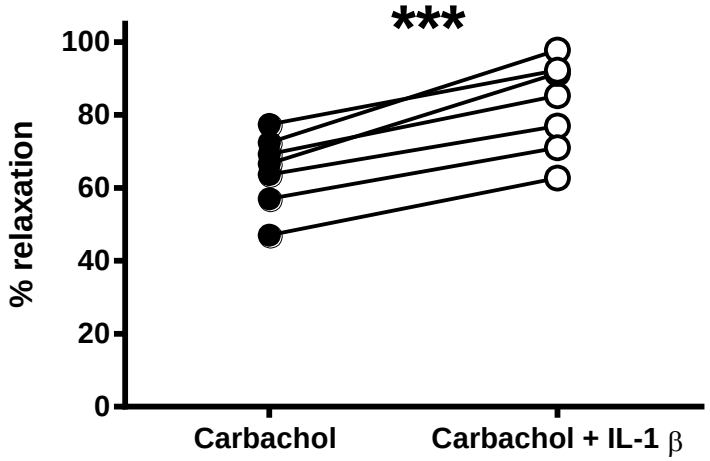

Figure 6.1. The effect of the IL-1β on relaxation to carbachol.
The relaxation to carbachol (64.8 % ± 3.8) was increased by the cytokine IL-1β (30pM) (82.5 % ± 4.8). Results show mean ± SE, n=7, *** $p < 0.001$

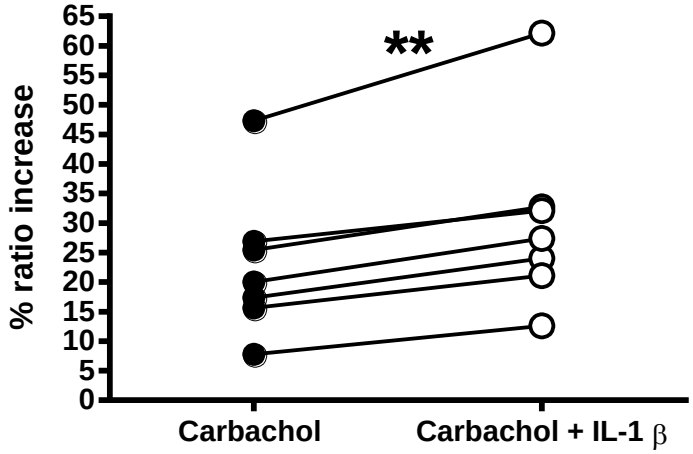

Figure 6.2. The effect of the IL-1β on the ratio increase to carbachol.
The 360/380nm ratio increase to carbachol (22.9 % ± 4.7) was potentiated by the cytokine IL-1β (30pM) (30.3 % ± 5.9). Results show mean ± SE, n=7, ** $p < 0.01$.

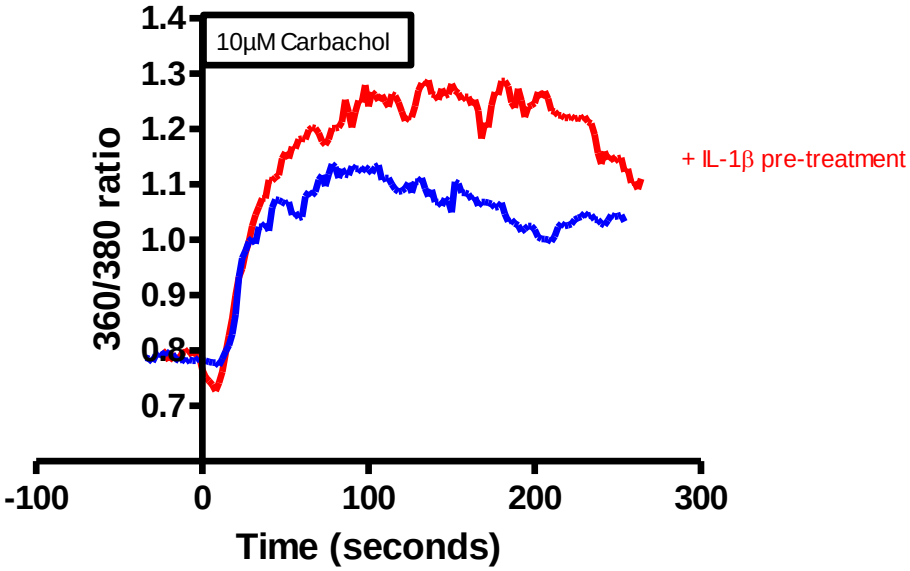

Figure 6.3. A typical trace of the 360/380nm ratio increase following carbachol application.
Pre-treatment with the cytokine IL-1β (30pM) enhanced the rise in Ca^{2+} to carbachol.

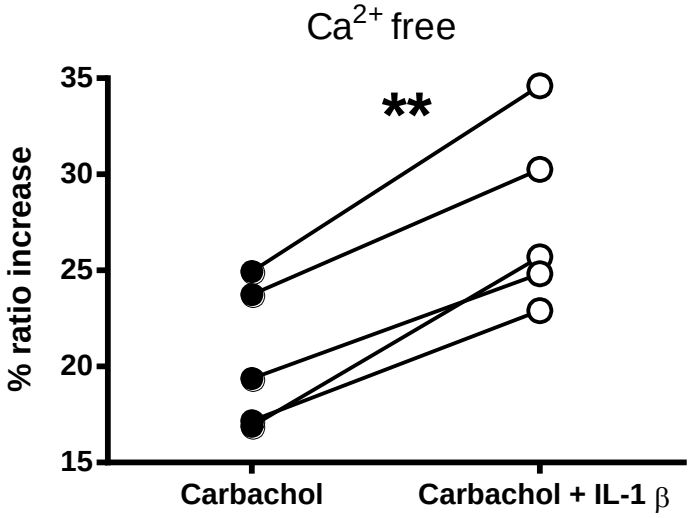

Figure 6.4. The effect of IL-1β on the ratio increase to carbachol in the Ca^{2+} free preparation.
The 360/380nm ratio increase to carbachol (20.4 % ± 1.7) was potentiated by the cytokine IL-1β (30pM) (27.7 % ± 2.1). Results show mean ± SE, n= 5, ** $p < 0.01$.

6.3.2 IL-1β mechanism

To investigate the mechanism behind the enhanced response when pre-treated with IL-1β, the ROS scavengers SOD and catalase were used. The antioxidants abolished the enhanced response and inhibited the EDHF response to carbachol suggesting that IL-1β was working via increased ROS (see Fig. 6.5 & 6.6).

To study whether the increase in ROS is due to an increase in NADPH oxidase activation, the inhibitor apocynin was used. Apocynin abolished the IL-1β-mediated enhanced response to carbachol but did not inhibit the base response (see Fig. 6.7 & 6.8). The PKC inhibitor Ro 31-8220 was also applied and this also inhibited the enhanced response (see Fig. 6.9 & 6.10). These results suggest that IL-1β was working via a mechanism that involved the NADPH oxidase pathway.

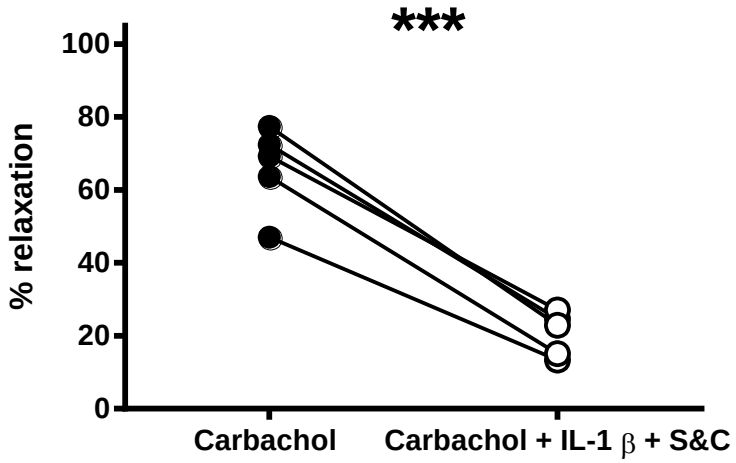

Figure 6.5. The effect of IL-1β when antioxidants are present on relaxation to carbachol.
The relaxation to carbachol (65.9 % ± 5.2) was inhibited when pre-treated with IL-1β (30pM) in the presence of the antioxidants SOD and catalase (100U/ml each) (20.6 % ± 2.7). Results show mean ± SE, n=5, *** $p < 0.001$.

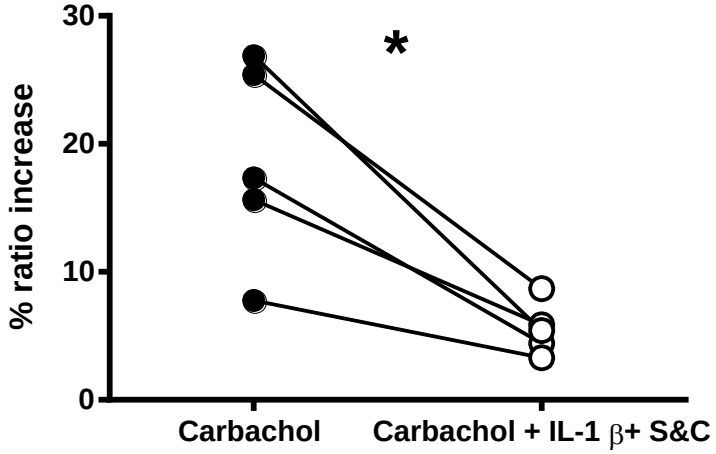

Figure 6.6. The effect of IL-1β when antioxidants are present on the relaxation to carbachol.
The 360/380nm ratio increase to carbachol (18.6 % ± 3.5) was inhibited by IL-1β (30pM) when the antioxidants SOD and catalase (100U/ml each) were present (5.5 % ± 0.9). Results show mean ± SE, n=5 , * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

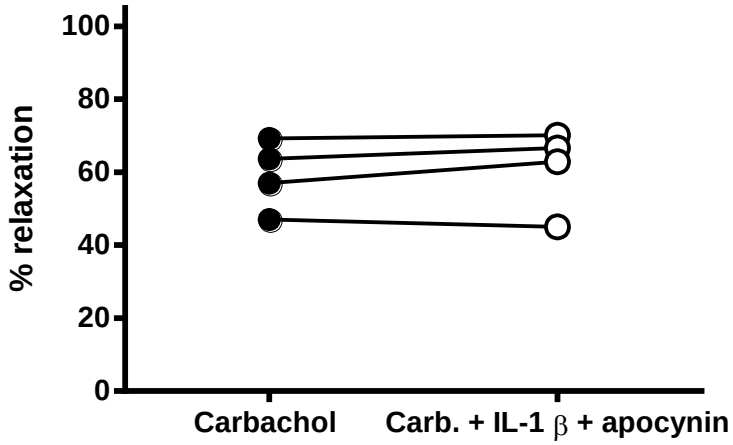

Figure 6.7. The effect of IL-1β on relaxation to carbachol in the presence of apocynin.
The relaxation to carbachol (59.2 % ± 4.8) was not affected by IL-1β (30pM) when the NADPH oxidase inhibitor apocynin (1μM) was present (61.2 % ± 5.6). Results show mean ± SE, n=4, (n.s).

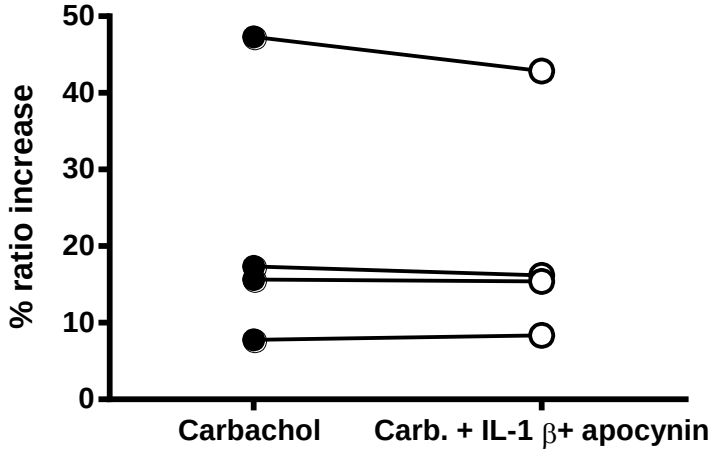

Figure 6.8. The effect of IL-1β on the ratio increase to carbachol in the presence of apocynin.
The 360/380nm ratio increase to carbachol (22.0 % ± 8.7) was not affected by the cytokine IL-1β (30pM) when the NADPH oxidase blocker apocynin (1μM) was present (20.7 % ± 7.6). Results show mean ± SE, n=4, (n.s).

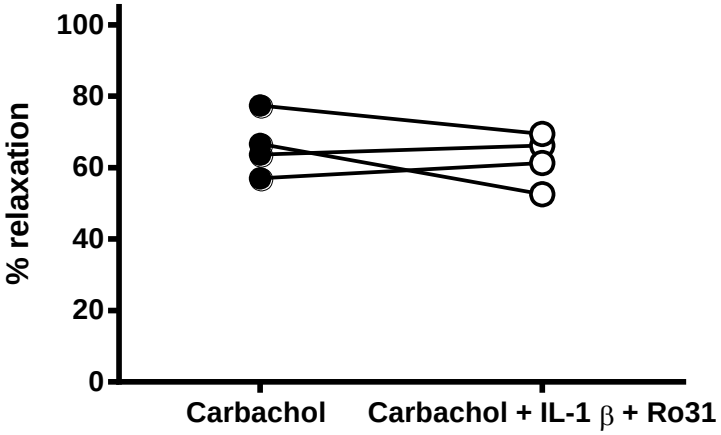

Figure 6.9. The effect of IL-1β on the relaxation to carbachol when PKC is inhibited.

The relaxation to carbachol (66.2 % ± 4.3) was not affected by IL-1β (30pM) pre-treatment when the PKC inhibitor Ro31-8220 (3μM) was present (62.4 % ± 3.7). Results show mean ± SE, n=4, (n.s).

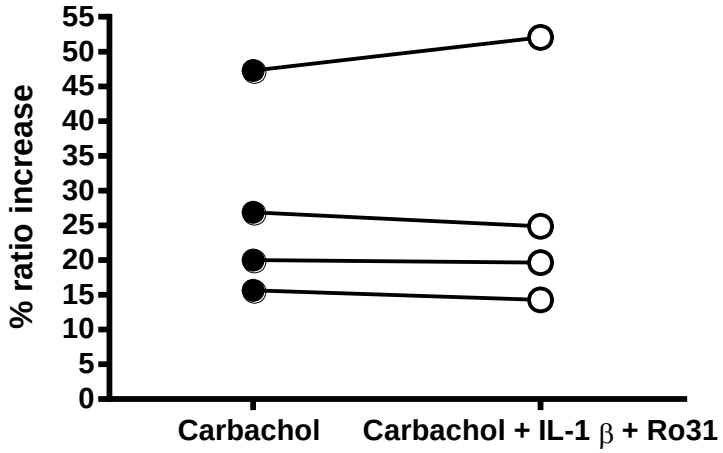

Figure 6.10. The effect of IL-1β on the ratio increase to carbachol when PKC is inhibited.

The 360/380nm ratio increase to carbachol (27.5 % ± 7.0) was not significantly affected by IL-1β (30pM) when the PKC inhibitor Ro31-8220 (3μM) was present (27.7 % ± 8.4). Results show mean ± SE, n=4, (n.s).

6.4 Discussion

6.4.1 IL-1β and the carbachol response

In previous chapters it has been shown that enhancing ROS levels, through exogenous H_2O_2, or blocking the breakdown of ROS, potentiates the EDHF response. IL-1β is a cytokine that can increase ROS production. When pre-treated with IL-1β, the EDHF response to carbachol was enhanced with an increase in relaxation and endothelial [Ca^{2+}]. In the Ca^{2+} free preparation, IL-1β also increased the response to carbachol, indicating that it was increasing the Ca^{2+} release from intracellular stores. When the antioxidants SOD and catalase were present, the EDHF response to carbachol was inhibited and IL-1β did not have any effect. This suggests that the response is through ROS production rather than a different mechanism.

6.4.2 IL-1β mechanism

The NADPH oxidase inhibitor apocynin and the PKC inhibitor Ro-31 8220 both blocked the enhanced IL-1β response to carbachol, but not the baseline response to carbachol. This supports the previous work that has shown that IL-1β works by increasing NADPH oxidase assembly and activation which then causes an increase in ROS production. It was shown in endothelial cells that IL-1β increased ROS production, and enhanced a ROS dependent mechanism, in that case increased permeability to bradykinin (Woodfin et al., 2011).

6.4.3 Summary

This is the first time IL-1β has been used to investigate the IL-1β response. IL-1β increased NADPH oxidase activation and ROS production. This enhanced the EDHF response to carbachol, partly by potentiating the Ca^{2+} release from intracellular stores in endothelial cells.

Chapter 7: Final discussion

7.1 Discussion

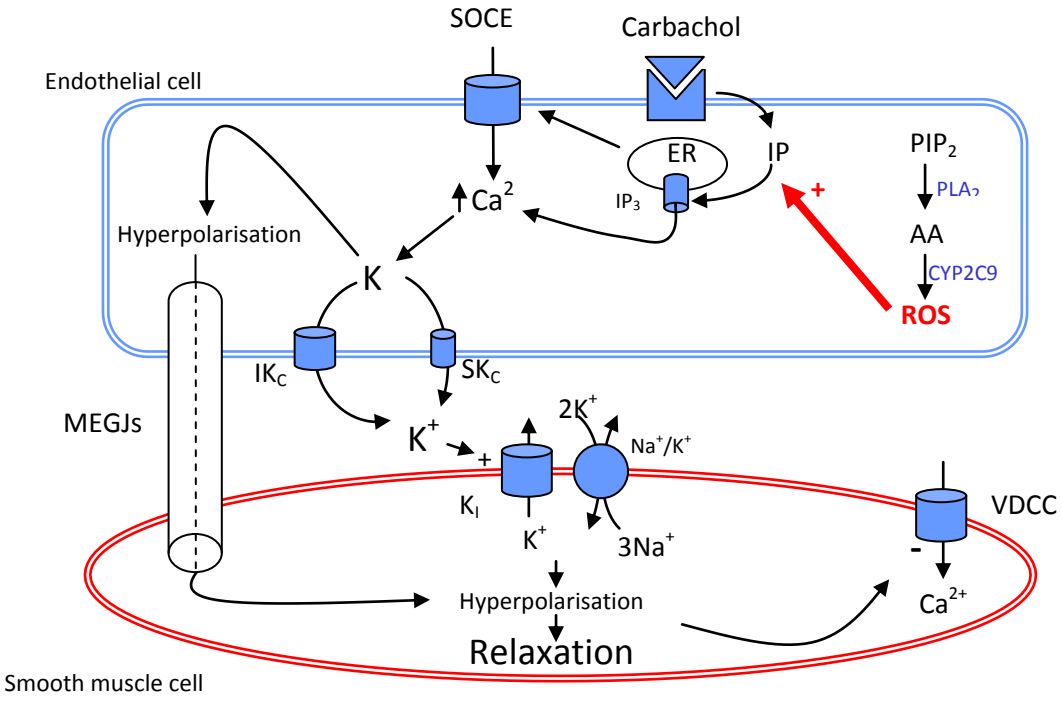

Figure 7.1. Scheme showing the pathway that mediates the EDHF relaxation to carbachol.

The results in this thesis have demonstrated an important role for ROS in the EDHF response in rat cremaster arterioles. The EDHF response to carbachol, measured by relaxation and the increase in endothelial [Ca^{2+}], was inhibited by antioxidants. The ROS appeared to act by enhancing the release of [Ca^{2+}] from intracellular stores. This increase in endothelial [Ca^{2+}] would potentiate the EDHF response by activating the IK_{Ca} and SK_{Ca} channels, leading to K^+ efflux and hyperpolarisation of the endothelial cell, this then leading to the hyperpolarisation and relaxation of the VSM. Since this relaxation was almost completely blocked by 18α-GA, it is likely that the EDHF-associated VSM relaxation under these conditions was primarily due to electrical coupling through the MEGJs in rat cremaster arterioles (see Fig. 7.1).

I found that a product of the pathway involving arachidonic acid production and metabolism, via PLA_2 and CYP2C9 respectively, was needed for the EDHF response

to carbachol. With the evidence that 11,12-EETs were not involved in the relaxation, taken with the results from the antioxidant experiments it seems likely that ROS are the active product. Indeed, when the EDHF response was inhibited using PLA_2 and CYP2C9 blockers, the response could be rescued with pre-treatment with H_2O_2 suggesting that it was loss of ROS that attenuated the response. This was supported by the Ca^{2+} release from intracellular stores and the EDHF response being enhanced by increased ROS levels, either through the addition of exogenous H_2O_2, or through increasing ROS production via activation of NADPH oxidase with the cytokine IL-1β.

Taken together, these results suggest that in rat cremaster arterioles ROS, produced by CYP2C9, potentiate the EDHF response by enhancing Ca^{2+} release from intracellular stores. This leads to hyperpolarisation of the endothelial cell which can spread via electrical coupling through MEGJs to the smooth muscle leading to relaxation.

7.1.1 Differences between H_2O_2 and carbachol responses.

The role of ROS during the EDHF response to carbachol was investigated. H_2O_2 is suspected to be the most likely species of ROS involved in cell signalling, and others have previously proposed that H_2O_2 is EDHF (Matoba et al., 2000). To study this directly, the effect of exogenous H_2O_2 in rat cremaster arterioles was looked at. 100μM H_2O_2 caused relaxation and a rise in endothelial [Ca^{2+}]. H_2O_2 appeared to be working through a number of mechanisms to cause relaxation (see Fig. 7.2). Carbachol and H_2O_2 worked through opening of the IK_{Ca} and SK_{Ca} channels, but while their inhibition almost abolished relaxation to carbachol it was only partially

blocked in the H_2O_2 mediated relaxation. Inhibition of the BK_{Ca} channels had no effect on the response to carbachol whereas it reduced the relaxation to H_2O_2 suggesting that H_2O_2 was acting directly on the BK_{Ca} channels. PKG inhibition also did not affect the EDHF response to carbachol while it did block the response to H_2O_2. This suggests that when a high concentration of exogenous H_2O_2 is added, it can cause relaxation by acting directly on mechanisms on VSM to cause hyperpolarisation and lower intracellular [Ca^{2+}]. However in the carbachol response, though ROS are involved, they are not acting directly on the VSM but rather on the release of Ca^{2+} from the endothelial stores as previously described. In rat coronary arteries exogenous H_2O_2 has also been shown to act through direct activation of PKG leading to relaxation using 100μM H_2O_2 (Burgoyne et al., 2007). Exogenous H_2O_2 has been demonstrated to increase the opening of the BK_{Ca} channel on smooth muscle as well in path clamp studies in rat mesenteric artery (Bryan et al., 2005). However, it has also been shown that BK_{Ca} channel activation occurs under normal conditions without the addition of exogenous H_2O_2. In human coronary arterioles, the EDHF response to shear stress was iberiotoxin and catalase sensitive suggesting that H_2O_2 was opening BK_{Ca} channels (Liu et al., 2011). These differing results between tissues could be due to a concentration dependency of the effect of H_2O_2, or through different tissue distribution of channels as BK_{Ca} was not involved in the EDHF response to carbachol in the rat cremaster arterioles.

Use of the TRPM2 antibody to block the redox sensitive TRPM2 channel reduced the rise in endothelial [Ca^{2+}] following H_2O_2 application, but had no effect on the EDHF response to carbachol. This activation of the TRPM2 channel by H_2O_2 in endothelial cells has been shown by others with the H_2O_2 increasing the cation current following

application H_2O_2 (Hecquet et al 2008). This could be a concentration dependent effect as in this study and the study by Hecquet et al., 100µM was needed to open the TRPM2 channels so this may not be relevant under physiological conditions where [H_2O_2] is likely to be much lower.

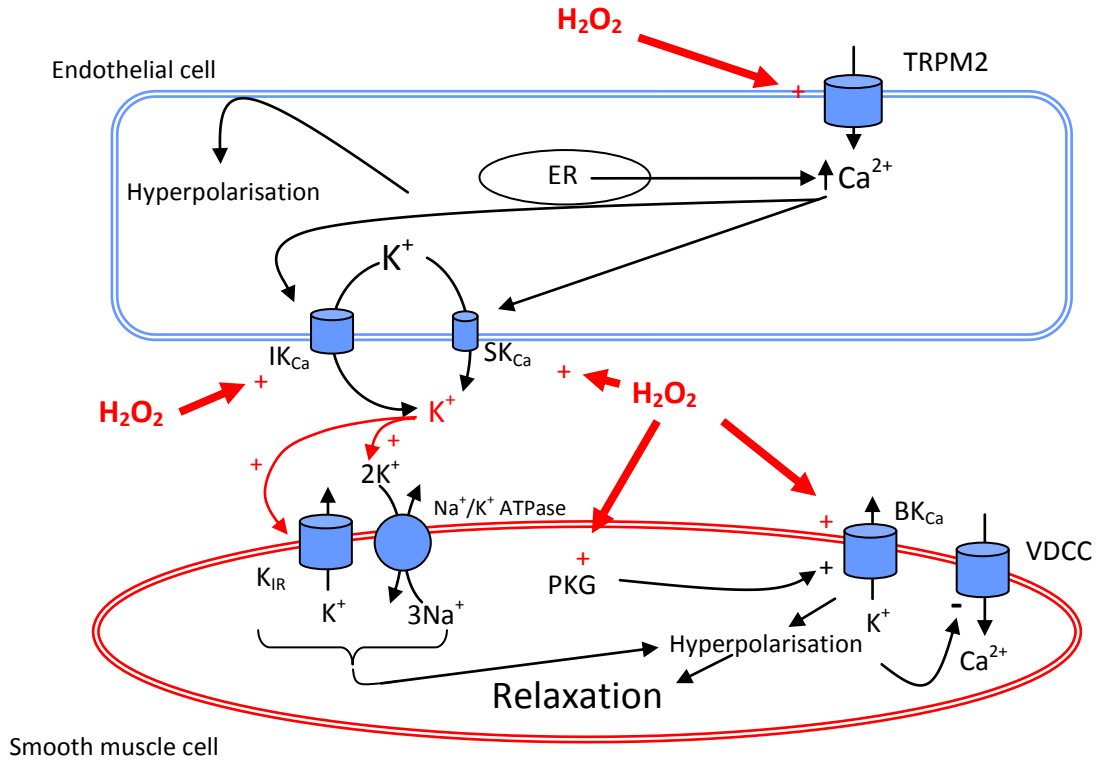

Figure 7.2. Scheme showing the mechanisms that mediate the relaxation to H_2O_2.

The differences between the response to carbachol and the response to H_2O_2 could explain some of the variation in results in previous studies. This study demonstrates a role for ROS in the EDHF response, and lower concentrations of H_2O_2 could potentiate the response to carbachol and rescue it when ROS production was blocked. However, unlike with exogenous H_2O_2, the ROS in the carbachol response appeared to be acting on the release of intracellular Ca^{2+} in the endothelial cells, rather than directly on the VSM. This may explain why in studies with exogenous H_2O_2, it has been suggested to be EDHF, produced by the endothelium and then activating BK_{Ca}

channels and other mechanisms on the smooth muscle leading to relaxation (Barlow et al., 1998; Matoba et al., 2001). Others however have found that H_2O_2 was not acting on the VSM, as it had no effect in endothelium denuded arteries (Hatoum et al., 2005). While this could be partly due to species and tissues differences, it could also be due to a concentration dependent effect with potentially erroneous conclusions being drawn from experiments using high exogenous levels of H_2O_2. The results of this study are therefore important as they demonstrate a difference between the mechanisms of exogenous H_2O_2, and the EDHF response to carbachol where ROS levels are likely to be lower and more regulated (compare Fig. 7.1 & 7.2).

7.1.2 ROS signalling

This study has demonstrated the important of ROS in the EDHF response. They appear to be acting through the release of Ca^{2+} from endothelial stores, possibly through the IP_3R. While it was not investigated in this study, this could be through the redox medication of the IP_3 receptor. This has been suggested previously the mechanism by which H_2O_2 can potentiate the EDHF response (Edwards et al., 2008). H_2O_2 can selectively target thiol groups on cysteine residues. The oxidation of the groups can lead to a conformational change in the structure of the protein which can affect its functioning (Janssen-Heininger et al., 2008). This could sensitise the IP_3 receptor to IP_3 activation which would enhance the response (Hidalgo et al., 2008). This would mean following simulation by an agonist such as carbachol there would be an increase in Ca^{2+} release from the stores which would potentiate the EDHF response (Hu et al., 2002). This study is therefore important as it supports the growing

evidence for ROS having a vital role in physiological cell signalling as in rat cremaster arterioles it is part of the regulation of vascular tone.

7.1.3 The EDH factor

Since the discovery of a third endothelium dependent pathway, many studies have looked for a universal endothelium dependent hyperpolarising factor (EDHF). This study was looking at the role of ROS in the EDHF response to carbachol. It was found that CYP2C9 derived ROS, potentiated the EDHF response to carbachol. They did this by increasing the release of Ca^{2+} from endothelial stores. CYP2C9 has been shown to be a source of ROS in endothelial cells and metabolites of CYP450 pathway have been demonstrated to be involved in EDHF mediated relaxation (Bolz et al., 2000; Fleming et al., 2001). However, this is the first time that CYP2C9 derived ROS have been shown to be involved in the EDHF response. This study supports the growing evidence that rather than there being a single universal EDH factor, that EDHF is a process that leads to hyperpolarisation of the smooth muscle cell (Griffith, 2004). This involves an increase in endothelial cell [Ca^{2+}] which opens the IK_{Ca} and SK_{Ca} channels leading to hyperpolarisation of the endothelial cell. This then cause the hyperpolarisation and relaxation of the smooth muscle cell. In this study, electrical coupling through MEGJs appeared to be involved, but at lowers levels of excitation it is possible that that K^+ in the myoendothelial space activates the Na^+/K^+ATPase leading to hyperpolarisation of the smooth muscle cell (Mather et al., 2005, Edwards et al., 2010). This, however, remains to be established.

In conclusion, CYP2C9 derived ROS act as cell signalling molecules in the EDHF response to carbachol in rat cremaster arterioles. Rather than being the EDH factor itself, the ROS potentiate the EDHF response by increasing endothelial cell Ca^{2+} release from stores. This study provides further evidence of the important role of ROS in cell signalling through redox modification of proteins, and demonstrates how ROS are a key part of the EDHF relaxation pathway.

7.1.4 Limitations

This study primarily focused on the use of intravital microscopy to measure changes in vessel diameter and endothelial [Ca^{2+}] during the EDHF response. This limited some of the experiments that could take place. Ideally ROS measurements would have been carried out to confirm the source of the ROS and show the increase during the EDHF response. However it was not possible to successfully load the cells with a ROS indicator despite attempts using several different protocols and indicators. This problem has been found by others where high levels of exogenous H_2O_2 (100μM) were needed to increase fluorescence so changes at physiological levels could not be measured (Edwards et al., 2008). This could be partly due to the tissue being studied in situ while most studies where ROS have been successfully measured were in cultured cells (Dickinson et al., 2010).

Therefore, a primarily pharmacological approach was used to investigate the mechanisms involved in the EDHF response. While the use of inhibitors followed protocols used in previous studies, antagonists can often have varying selectivity. Where possible the inhibitor with the greatest selectivity for its target was chosen, and

I also used multiple inhibitors where these were available. However, there still could be interactions of these drugs with other receptors and channels which could influence the results.

A strict experimental protocol was followed so that there were no confounding factors in the experiments. However, a limitation of using this model with rat tissue is that there will be individual variation between animals. This could be effected by sleep or stress levels prior to the study. Also while drugs were sourced from the same companies each time, there could be possible difference in activity between batches. The effects of these confounding factors were limited by the use of the same animals as they control, with all data paired. This should ensure that the comparison between results on the same vessel are reliable.

A further limitation was that only arterioles in one tissue bed were investigated. This limits the conclusions that can be drawn as from other studies it appears that there are tissue and species differences, possibly because of variation in channel and receptor expression (Samora et al., 2008).

7.1.5 Further work

A number of further types of experiment could be carried out to support and extend the findings of this study.

The importance of the described pathway could be investigated in other tissues and species, ideally in human arteries. This would show if the results are reproducible and

generalisable, and highlight the potential importance of the ROS signalling pathway in other tissues.

KO mice could be used to provide greater selectivity than the pharmacological approach used. Firstly the importance of the pathway would have to be demonstrated in wild type mice. The effect on the EDHF response in a CYP2C9 KO mice would be able to support its role in ROS production. Also, further studies that could successfully use ROS indicators to demonstrate an increase in ROS levels in response to carbachol would support the finding that it is ROS that are the active metabolite.

Further work could investigate the exact target of the ROS. This could show if redox modification of the thiol group on the IP_3 receptor is the downstream target of the ROS. The use of a different agonist to pre-constrict the arterioles, or using a lower concentration could be carried out. This would investigate whether the contribution of MEGJs is reduced while K^+ becomes the main signalling mechanism between the endothelium and smooth muscle. This has been shown when there are lower levels of excitation (Dora et al., 2008). The experiments could also be reproduced using different stimuli such as bradykinin, or the more physiologically relevant shear stress. Demonstrating that this pathway is involved in the EDHF response to flow would show that this mechanism is relevant in physiological conditions. This could also be investigated in disease models such as diabetes to see if there is variation in the signalling pathway and the EDHF response.

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